# ROLE OF MACROPHAGE MIGRATION INHIBITORY FACTOR (MIF) AND MIF PROMOTER POLYMORPHISMS IN THE PATHOGENESIS OF SEVERE MALARIAL ANEMIA

by

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**ROLE OF MACROPHAGE MIGRATION INHIBITORY FACTOR (MIF) AND MIF PROMOTER POLYMORPHISMS IN THE PATHOGENESIS OF SEVERE MALARIAL ANEMIA**

Gordon Akanzuwine Awandare, PhD

University of Pittsburgh, 2007

Severe malarial anemia (SMA), caused by infections with *Plasmodium falciparum*, is one of the leading causes of childhood mortality in sub-Saharan Africa. Although the molecular determinants of SMA are largely undefined, dysregulation in host-derived inflammatory mediators influences disease severity. Macrophage migration inhibitory factor (MIF) is an important regulator of innate inflammatory responses that has recently been shown to suppress erythropoiesis and promote pathogenesis of SMA in murine models. The role of MIF in childhood malarial pathogenesis was investigated by examining peripheral blood MIF production in children residing in a hyperendemic area of Gabon, and a holoendemic region of western Kenya. The relationship between MIF concentrations and monocytic acquisition of hemozoin, and the effects of MIF on erythropoiesis *in vivo* and *in vitro* were investigated. In addition, the influence of genetic variation at MIF -173 (G/C) and -794 ($CATT_{5-8}$) on MIF production and susceptibility to SMA and high-density parasitemia (HDP) was examined. Circulating MIF concentrations and peripheral blood mononuclear cells (PBMC) MIF production progressively declined with increasing anemia severity and increasing levels of hemozoin-containing monocytes. However, circulating MIF concentrations were not significantly associated with reticulocyte production in children with acute malaria. Additional experiments in malaria-naïve individuals demonstrated that hemozoin caused both increased and decreased MIF production in cultured PBMC based on genetic differences. In addiiton, a novel *in vitro* model of

erythropoiesis was developed and used to demonstrate that treatment with exogenous MIF or blocking endogenous MIF did not signifcantly impact on the efficiency of erythropoiesis. Genetic analyses revealed that the MIF -173 CC genotype was associated with an increased risk of HDP compared to MIF -173 GG. In addition, individuals with the MIF -794CATT$_6$/-173G haplotype were significantly protected from SMA while those with -794CATT$_{7/8}$/-173C haplotypes were at an increased risk of developing SMA. Taken together, our findings demonstrate that SMA is associated with decreased MIF production and that individuals with high MIF-producing genetic variants are less susceptible to severe malaria. The public health significance of this study is that investigations presented here increase our understanding of protective inflammatory responses to childhood malaria, which is critical in the formulation of an effective malarial vaccine.

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## PREFACE

The results of investigations conducted for this dissertation are presented as manuscripts that have either been published, or are ready to be submitted for publication. Permission has been obtained from all co-authors and from publishers of all the journals in which this work has been published. The following is the full list of manuscripts presented in this dissertation:

1. Gordon A. Awandare, James B. Hittner, Peter G. Kremsner, Daniel O. Ochiel, Christopher C. Keller, Brice J. Weinberg, Ian A. Clark, and Douglas J. Perkins. Decreased circulating macrophage migration inhibitory factor (MIF) protein and blood mononuclear cell MIF transcripts in children with *Plasmodium falciparum* malaria. *Clinical Immunology 2006*. 119: 219-225. Reproduced with permission from Elsevier Limited, UK.
2. Gordon A. Awandare, James B. Hittner, Peter G. Kremsner, Daniel O. Ochiel, Christopher C. Keller, Brice J. Weinberg, Ian A. Clark, and Douglas J. Perkins. Higher production of peripheral blood macrophage migration inhibitory factor in healthy children with a history of mild malaria relative to children with a history of severe malaria. *American Journal of Tropical Medicine and Hygiene*, *2007.* 76(6): 1033-1036. Reproduced with permission from the American Journal of Tropical Medicine and Hygiene, USA.

3. Gordon A. Awandare, Yamo Ouma, Collins Ouma, Tom Were, Richard Otieno, Christopher C. Keller, Gregory C. Davenport, James B. Hittner, John Vulule, Robert Ferrell, John M. Ong'echa, and Douglas J. Perkins. Role of monocyte-acquired hemozoin in suppression of macrophage migration inhibitory factor in children with severe malarial anemia. *Infection and immunity, 2007*. 75(1): 201-210. Reproduced with permission from the American Society of Microbiology, USA.

*4.* Gordon A. Awandare, Daniel O. Ochiel, Paolo Piazza, Christopher C. Keller, Douglas J. Perkins. An investigation of the roles of malaria-associated inflammatory mediators macrophage migration inhibitory factor (MIF), tumor necrosis factor (TNF)-α, and nitric oxide (NO) in regulation of erythropoiesis using a novel *in vitro* model. *Manuscript-in-preparation.*

5. Gordon A. Awandare, Collins Ouma, Christopher C. Keller, Tom Were, Richard Otieno, Yamo Ouma, Gregory C. Davenport, James B. Hittner, John M. Ong'echa, Robert Ferrell, and Douglas J. Perkins. A macrophage migration inhibitory factor promoter polymorphism is associated with high-density parasitemia in children with malaria. *Genes and Immunity, 2006. 7(7):568-575*. Reproduced with permission from Nature Publishing Group, UK.

*6.* Gordon A. Awandare, Jeremy Martinson, Collins Ouma, Gregory Davenport, John Michael Ong'echa, Robert Ferrell, Richard Bucala, and Douglas J. Perkins. Macrophage migration inhibitory factor promoter haplotypes are associated with susceptibility to severe malarial anemia in children. *Manuscript-in-preparation.*

## LIST OF ABBREVIATIONS

AC, aparasitemic control

ARN, absolute reticulocyte number

CM, cerebral malaria

CI, confidence interval

CD, cluster of differentiation

DMEM, Dulbecco's modified Eagle's medium

EU, endotoxin unit

ELISA, enzyme-linked immunosorbent assay

EDTA, ethylenediaminetetraacetic acid

Epo, erythropoietin

ERK, extracellular regulated kinase

FP, ferriprotoporphyrin

GPA, glycophorin-A

GPI, glycosylphosphatidylinositol

HC, healthy control

GITC, guanidinium isothiocyanate

Hb, hemoglobin

HDP, high-density parasitemia

HIV, human immunodeficiency virus

Hz, hemozoin

HWE, Hardy Weinberg equilibrium

Ig, immunoglobulin

IFN, interferon

IL, interleukin

IMDM, Iscove's modified Dulbecco's medium

LDP, low-density parasitemia

LPS, lipopolysaccharide

MIF, macrophage migration inhibitory factor

MIP, macrophage inflammatory protein

MHC, major histocompatibility complex

MdMA, moderate malarial anemia

MlMA, mild malarial anemia

MTT, methylthiazoletetrazolium

NO, nitric oxide

NOS, nitric oxide synthase

OR, odds ratio

PBMC, peripheral blood mononuclear cell

PBL, peripheral blood leukocytes

PBS, phosphate buffered saline

PCM, pigment-containing monocytes

PCN, pigment-containing neutrophils

PCR, polymerase chain reaction

*Pf*Hz, *Plasmodium falciparum*-derived hemozoin

PG, prostaglandin

PMM, prior mild malaria

pRBC, parasitized red blood cell

PSM, prior severe malaria

RBC, red blood cell

RFLP, restriction fragment length polymorphism

rh, recombinant human

RT, reverse transcription

SCF, stem cell factor

sHz, synthetic hemozoin

SMA, severe malarial anemia

SNP, single nucleotide polymorphism

SEM, standard error of the mean

STRP, short tandem repeat polymorphism

TLR, toll-like receptor

TGF, transforming growth factor

TNF, tumor necrosis factor

UM, uncomplicated malaria

WBC, white blood cell

WHO, World Health Organization

# 1.0 CHAPTER ONE: INTRODUCTION

Malaria continues to be a major global health concern, with an estimated 3.2 billion people across over 100 countries at risk of the disease (WHO 2005). There are 350-500 clinical cases of malaria annually, resulting in about 2 million deaths around the world (WHO 2005). The majority of malaria morbidity occurs in Africa (~60%), which also accounts for 89% of the global malaria-related mortality (WHO 2003). In sub-Saharan Africa, malaria is one of the leading causes of childhood morbidity and mortality, being responsible for 25-35% of outpatient visits, 20-45% of hospital admissions, and up to 35% of inpatient deaths (WHO 2005). In addition to directly causing 18% of all-cause mortality in children under five years old, malaria indirectly contributes to mortality through maternal anemia in pregnancy, low birth weights, pre-term deliveries, and increased susceptibility and severity of other infections, such as bacteremia and human immunodeficiency virus (HIV) (Molineaux 1997; WHO 2005).

## 1.1 MALARIA PARASITE

Malaria is caused by infections of a protozoan parasite of the genus *Plasmodium*. Four species of the genus infect humans, namely, *P. vivax, P. ovale, P. malariae* and *P. falciparum*. The vast majority of malaria morbidity worldwide, and greater than 90% of malaria-related mortality in

sub-Saharan Africa result from *P. falciparum* infections (WHO 2005). *P. falciparum* has a complex life cycle, requiring an invertebrate host, a female *Anopheles* mosquito, in addition to the human host for complete replication. The parasite's life cycle includes a cycle of asexual division in the human liver, another cycle of pigment-producing asexual division in red blood cells (RBCs), and a sporogenic development in the mosquito. The cycle begins with the bite by an infected mosquito inoculating the parasites in the form of sporozoites into the bloodstream of the human host (Figure 1). After circulating in the bloodstream for not more than 1 hour, the sporozoites enter hepatocytes, where the parasites grow, multiply and develop directly into pre-erythrocytic (PE) schizonts. These schizonts take 5-7 days to mature, culminating in the rupture of the hepatocyte and release of merozoites into bloodstream to invade RBCs. Successful entry of the parasites into RBCs starts the erythrocytic cycle, which takes 36-48 hours to complete. Within the RBC, the merozoite develops into a trophozoite in a vacuole formed by the internal membrane of the host red cell. The trophozoite feeds on hemoglobin (Hb) by ingesting small amounts of red cell cytoplasm, which leads to accumulation of malaria pigment, hemozoin (Hz), as an end product of Hb breakdown. Fully matured trophozoites divide to form schizonts containing 8-32 merozoites, which rupture to release the merozoites, hemozoin and toxins into the blood stream. The initiation of the systemic immune response, and the appearance of clinical symptoms such as fever, coincide with schizont rupture (Kwiatkowski *et al.* 1989; Kwiatkowski and Perlman 1999). Merozoites that escape the host's immune system re-invade new RBCs to begin another replication cycle. Occasionally, some trophozoites develop into male and female gametocytes which can be taken up by the mosquito in its blood meal. Gametocytes differentiate into gametes in the midgut of the mosquito, and fertilization takes place to form a zygote. The zygote then develops into a motile ookinete, which penetrates the gut epithelium and matures to

form the oocyst containing large numbers of sporozoites. Mature sporozoites leave the oocytes and some find their way into the salivary gland of the mosquito, ready to be transmitted when the insect takes its next blood meal.

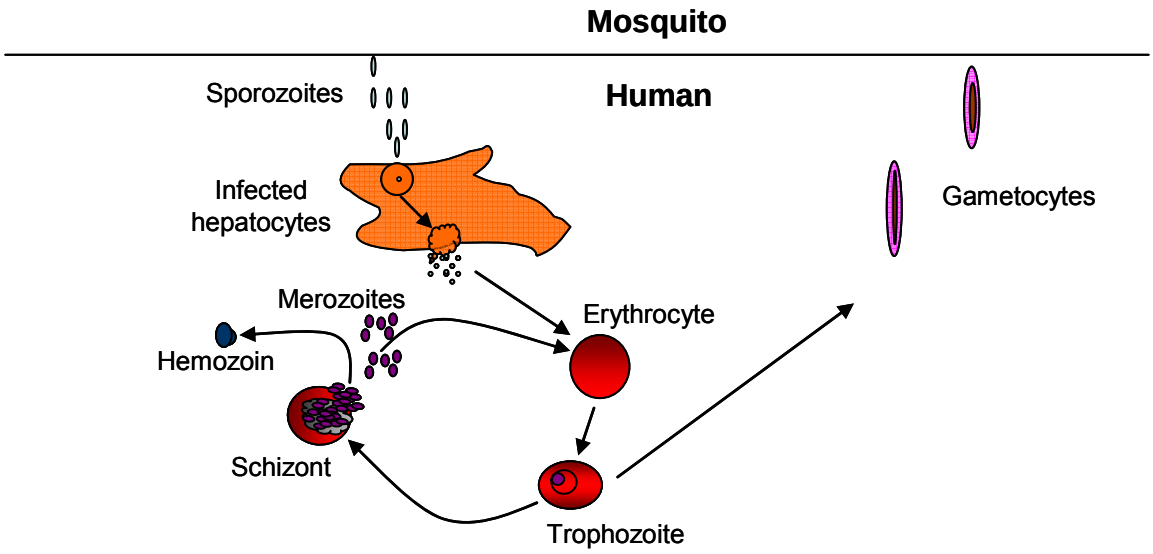


**Figure 1: *P. falciparum* life cycle in humans.**

Sporozoites injected into the blood stream during a bite by an infected mosquito find their way to the liver, where they infect hepatocytes. The parasites then mature into pre-erythrocytic schizonts which burst to release merozoites into the blood stream. These merozoites quickly invade erythrocytes, forming trophozoites which feed on host cell hemoglobin and grow rapidly. Matured trophozoites divide into many daughter merozoites while developing into a schizont which also contains hemozoin as a by-product of hemoglobin degradation. Rupture of mature schizonts releases hemozoin, and many merozoites to re-invade new erythrocytes and repeat the replicative cycle. Occasionally, some parasites develop into male and female gametocytes which can be taken up by the mosquito in a subsequent blood meal.

## 1.2 CLINICAL MANIFESTATIONS OF MALARIA

The clinical features of *P. falciparum* infection cover a wide spectrum from asymptomatic to fulminant disease leading to death. The vast majority of cases present as a relatively mild, non-specific febrile illness, which resolves rapidly if treated appropriately, however, some children develop severe, life-threatening complications. The classical severe malarial presentations responsible for most of the malaria-associated mortality in children are severe anemia (Hb levels <5.0 g/dL), and cerebral malaria characterized by neurological symptoms, including deep coma (WHO 2000). Other important features of severe malaria include hyperparasitemia, metabolic acidosis, respiratory distress, and hypoglycemia (WHO 2000). Clinical presentations of pediatric severe malaria vary markedly across regions with differing transmission intensities, with CM primarily occurring in lower transmission regions and SMA being most prevalent in holoendemic areas (Snow *et al.* 1997). Additional determinants of the clinical pattern of malaria include age, immune status, genetic factors, and parasite species, however, the relative contributions of individual factors in disease pathogenesis remains unknown.

## 1.3 PATHOGENESIS OF SEVERE MALARIAL ANEMIA (SMA)

The pathogenesis of SMA is multifactorial and not well understood. Some of the earliest studies of SMA identified three categories of patients: children with *acute* infection associated with RBC hemolysis, and who had the most marked anemia during the period after treatment; a second group of children appeared to have a more *chronic* form of *P. falciparum* infection, were profoundly anemic at presentation, showed gross dyserythropoietic changes in their bone marrows, and had a full reticulocyte response and rise in Hb after treatment; and a third group of children whose hematological abnormalities were *intermediate* to those of the acute and chronic groups (Abdalla *et al.* 1980). Although the relative contributions of each of the potential mechanisms that promote anemia are not clear, three processes are involved in the development of SMA: direct and indirect destruction of parasitized red blood cells (pRBCs); increased clearance of uninfected erythrocytes; and suppression of erythropoiesis (Wickramasinghe and Abdalla 2000). Destruction of red cells seems to occur both intravascularly via immune-mediated mechanisms and parasite replication, and by sequestration of parasitized cells in the spleen and other parts of the microcirculation resulting from parasite adherence, increased rigidity and reduced deformability (Weatherall and Abdalla 1982). The significance of the loss of uninfected RBCs in promoting SMA is demonstrated by studies showing that severe anemia is common even in individuals in which less than 1% of their erythrocytes are infected (Looareesuwan *et al.* 1987). Although the mechanism(s) by which unparasitized RBCs are destroyed is not clear, RBC survival studies demonstrate that both endogenous RBCs and transfused normal erythrocytes have shortened survival during human *P. falciparum* malaria

(Looareesuwan *et al.* 1987), suggesting that the existing inflammatory milieu may be detrimental to RBC survival. Development of SMA in children is also enhanced by a defective erythropoietic response, resulting in an inadequate production of reticulocytes to replace lost RBCs. Defective reticulocyte production during malaria is caused by depression of erythropoiesis, inhibition of reticulolyte release, and/or dyserythropoiesis (Rencricca *et al*. 1974) (Woodruff *et al.* 1979; Abdalla *et al.* 1980). Findings by Kurtzhals *et al*. suggested that *P. falciparum* infection caused a rapidly reversible suppression of the bone marrow response to erythropoietin (Kurtzhals *et al.* 1997). In addition, children with SMA show marked dyserythropoietic changes, including multinuclear erythroblasts, karyorrhexis, incomplete and unequal amitotic nuclear divisions, and cytoplasmic bridging (Abdalla *et al.* 1980). Although the molecular mechanisms that interfere with the erythropoietic process during malaria is not clear, deposition of Hz and over-production of pro-inflammatory mediators seem to contribute to suppression of erythropoiesis (Wickramasinghe and Abdalla 2000; Clark and Cowden 2003; McDevitt *et al.* 2004; Casals-Pascual *et al.* 2006).

## 1.4 ROLE OF HEMOZOIN IN MALARIAL PATHOGENESIS

During the erythrocytic stage of development, the malaria parasite forms a food vacuole in which host cell Hb is degraded into heme and globin. The globin component is further digested into amino acids for the parasite's metabolic needs; however, heme is toxic to the parasite and is thus aggregated into the insoluble dark-brown crystal, Hz (Figure 2). The immune response to blood stage malaria is induced by the repeated exposure to large quantities of parasite

products/antigens as matured parasites (schizonts) rupture and release young merozoites, Hz, and soluble antigens including glycosyl phosphatidyl inositols (GPIs). Whilst most soluble parasite antigens released during schizont rupture circulate for only a limited time, Hz persists for several days even after parasite clearance (Metzger *et al.* 1995; Day *et al.* 1996). Both circulating and resident phagocytes acquire Hz through phagocytosis of pRBCs or free Hz crystals released after schizont rupture ((Schwarzer *et al.* 1992), Figure 2). Extensive deposition of Hz has been observed in the livers and bone marrows of children with SMA, and associated with ultrastructural abnormalities in these tissues, suggesting a role in disease pathogenesis (Abdalla 1990; Wickramasinghe and Abdalla 2000; Giribaldi *et al.* 2004). Furthermore, several studies have demonstrated that acquisition of malarial pigment by circulating monocytes and neutrophils is significantly associated with disease severity (Nguyen *et al.* 1995; Luty *et al.* 2000; Lyke *et al.* 2003; Casals-Pascual *et al.* 2006). In addition, previous investigations from our laboratory, as well as those of others, show that ingestion of Hz by monocytes may enhance malarial pathogenesis by causing dysregulation in production of cytokine, chemokines, and effector molecules, including tumor necrosis factor (TNF)-α, interleukin (IL)-12, IL-10, macrophage inflammatory protein (MIP)-1α, MIP-1β, nitric oxide (NO), and prostaglandin (PG)-$E_2$ (Pichyangkul *et al.* 1994; Sherry *et al.* 1995; Perkins *et al.* 2003; Keller *et al.* 2004a; Keller *et al.* 2004b; Ochiel *et al.* 2005; Keller *et al.* 2006a; Keller *et al.* 2006b). In addition, acquisition of Hz by macrophages impairs their ability to repeat phagocytosis, and inhibits antigen presentation by down-regulating major histocompatibility (MHC)-II molecule expression (Schwarzer *et al.* 1992; Arese and Schwarzer 1997) .

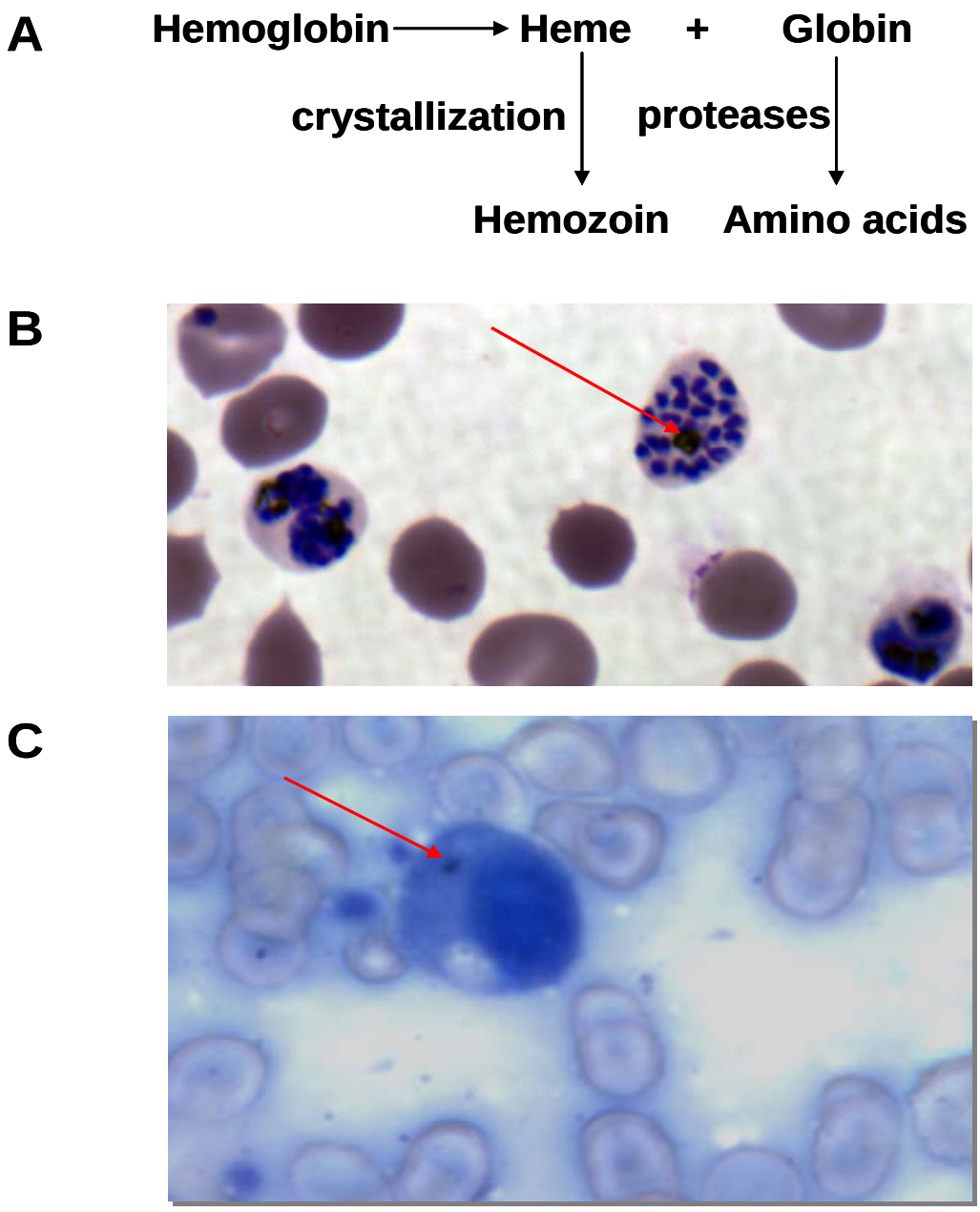


**Figure 2:** ***Hemozoin formation and acquisition by monocytes.***

Cleavage of host hemoglobin by the malaria parasite yields heme which is detoxified by crystallization into hemozoin, and globin which is digested into amino acids by parasitic proteases (A). Hz accumulates within the parasitized RBCs (pRBCs) and can be seen as a dark-brown pigment in the matured schizont (B). Monocytes acquire Hz through phagocytosis of pRBCs or free Hz released during schizonts rupture (C).

## 1.5 ROLE OF THE INNATE IMMUNE RESPONSE IN MALARIAL PATHOGENESIS

An integral part of a protective immune response to malarial infection is induction of interferon (IFN)-γ by IL-12; IFN-γ mediates control of parasitemia by activating monocytes/macrophages to undertake phagocytosis and secrete anti-plasmodial mediators including TNF-α and NO (Gyan *et al.* 1994; Stevenson and Riley 2004). This mechanism is supported by our previous studies, as well as those of others, showing that increased production of IL-12 and IFN-γ is associated with protection from childhood malaria (Luty *et al.* 1999; Luty *et al.* 2000; Perkins *et al.* 2000; John *et al.* 2004; Moormann *et al.* 2006). In addition, production of TNF-α may contribute to the protective immune response to malaria by inducing fever that is detrimental to parasite development, and/or by stimulating effector cells (Kwiatkowski and Perlman 1999). The host response to malaria also includes the production of increased numbers of peripheral blood monocytes and tissue macrophages in the spleen and the liver (Arese *et al.* 1991). This is believed to be a result of activation and mobilization of blood monocytes, and suggests that the macrophage is a major immunologic effector cell in malaria. Mononuclear phagocytes are able to recognize and ingest pRBCs, and have the potential to dispose of up to 40-80% of the total red cell mass over the course of a few days (Arese *et al.* 1991). In addition to increased phagocytosis, macrophages and neutrophils produce toxic oxygen radicals, which participate in parasite killing (Dockrell and Playfair 1983).

Despite the important role in parasite clearance, immune activation and production of pro-inflammatory cytokines could contribute to SMA pathogenesis by inhibiting erythropoiesis

and reducing RBC survival (Clark and Chaudhri 1988; Angulo and Fresno 2002; Clark and Cowden 2003; McDevitt *et al.* 2004). For example, various studies have shown that over-production of TNF-α, is associated with severe forms of malaria including SMA (Grau *et al.* 1989; Kwiatkowski *et al.* 1990; Kurtzhals *et al.* 1998; Akanmori *et al.* 2000; Luty *et al.* 2000; Perkins *et al.* 2000), suggesting an important role for this mediator in disease pathogenesis. Furthermore, production of reactive oxygen and nitrogen species may also contribute to SMA by causing increased destruction of uninfected RBCs and suppressing erythropoiesis (Clark *et al.* 1981; Shami and Weinberg 1996; Anstey *et al.* 1999; Keller *et al.* 2004b). In addition, IL-1β and IL-6 are elevated during *P. falciparum* infection, correlate with malaria disease severity, and are associated with enhanced anemia (Kern *et al.* 1989; Day *et al.* 1999; Vogetseder *et al.* 2004; Dinarello 2005; Ganz 2006; Prakash *et al.* 2006). Therefore, adequate induction of anti-inflammatory cytokines, such as IL-4, IL-10, and transforming growth factor (TGF)-β is required to counter-balance the pro-inflammatory response, and prevent the prolonged production of potentially harmful mediators (Ho *et al.* 1995; Ho *et al.* 1998; Omer and Riley 1998; Winkler *et al.* 1998; Omer *et al.* 2000; Stevenson and Riley 2004). Our previous studies, and those of others, demonstrate that the clinical course of severe malaria is influenced by the relative imbalance between TNF-α and IL-10 levels (Kurtzhals *et al.* 1998; Othoro *et al.* 1999; Akanmori *et al.* 2000; Perkins *et al.* 2000; Awandare *et al.* 2006a).

## 1.6 MACROPHAGE MIGRATION INHIBITORY FACTOR (MIF): BIOLOGICAL FEATURES

Macrophage migration inhibitory factor (MIF) is a 12.5 kDa protein of 114 amino acid residues (Donn and Ray 2004). Structural studies by X-ray crystallography show that MIF exists as a homotrimer (Sugimoto *et al.* 1996; Sun *et al.* 1996), however, bioactive monomeric and dimeric forms of MIF have been observed by other studies (Mischke *et al.* 1998). MIF was one of the first proteins described to have cytokine activity when it was originally identified for its role in delayed-type hypersensitivity, where it inhibited the random migration of macrophages (Bloom and Bennett 1966; David 1966). Although initially classified as a T cell-derived cytokine, recent studies have re-discovered MIF as a pleiotropic cytokine released by several other cell types, including monocytes/macrophages (Calandra *et al.* 1994), endothelial cells (Nishihira *et al.* 1998), and cells of the anterior pituitary gland (Bernhagen *et al.* 1993). Unlike most cytokines which are tightly regulated and induced only by stimulation, MIF is constitutively expressed at high levels and stored in vesicles, and therefore, can be rapidly released without de novo synthesis (Bernhagen *et al.* 1993; Bernhagen *et al.* 1998). MIF is also induced by various pro-inflammatory stimuli, including bacterial endo- and exotoxins such as lipopolysaccharide (LPS), and cytokines such as IFN-$\gamma$ and TNF-$\alpha$ (Calandra *et al.* 1994). Once released, MIF exerts potent pro-inflammatory properties by over-riding the immunosuppressive effects of glucocorticoids (Calandra *et al.* 1995; Calandra and Bucala 1995; Calandra *et al.* 2000), and inducing production of TNF-$\alpha$, NO, and IL-12 ((Calandra and Bucala 1995; Juttner *et al.* 1998), Figure 3). Studies in murine models of infection have demonstrated a central role for MIF in

regulating protective innate immunity to the bacterial pathogens such as *Salmonella enterica serovar Typhimurium* (Koebernick *et al.* 2002), and parasites including *Leishmania major* (Juttner *et al.* 1998), and *Trypanosoma cruzi* (Reyes *et al.* 2006), by activating macrophages for phagocytosis and cytokine production. Conversely, MIF exacerbates the pathogenesis of bacterial sepsis by promoting over-production of TNF-α, IL-6 and IL-1 (Calandra *et al.* 2000) (Calandra *et al.* 2003), demonstrating that MIF can elicit both protective and pathogenic immune responses to different infectious diseases.

MIF protein lacks a signal sequence, and as such, its mechanism of secretion is unclear. However, recent evidence suggests that synthesized MIF does not go to the endoplasmic reticulum, instead it is secreted via a non-classical export pathway involving an ATP binding cassette transporter ((Flieger *et al.* 2003), Figure 3). There is also ambiguity about the signal transduction pathways by which MIF exerts its biological activities, since MIF has no known receptors. The best known putative MIF receptor thus far is CD74, a type II transmembrane protein, that binds MIF with high affinity and appears to play a role in mediating MIF-induced activation of the extracellular signal-regulated kinase (ERK)-1/ERK-2 pathway ((Leng *et al.* 2003), Figure 3). A receptor-independent endocytic pathway for MIF signal transduction has also been proposed, following evidence showing co-localization of MIF with the JUN-activation domain-binding protein 1 (JAB1) in the cytoplasm. The interaction between MIF and JAB1 is believed to mediate biological activities such as cell growth, transformation and apoptosis ((Kleemann *et al.* 2000), Figure 3).

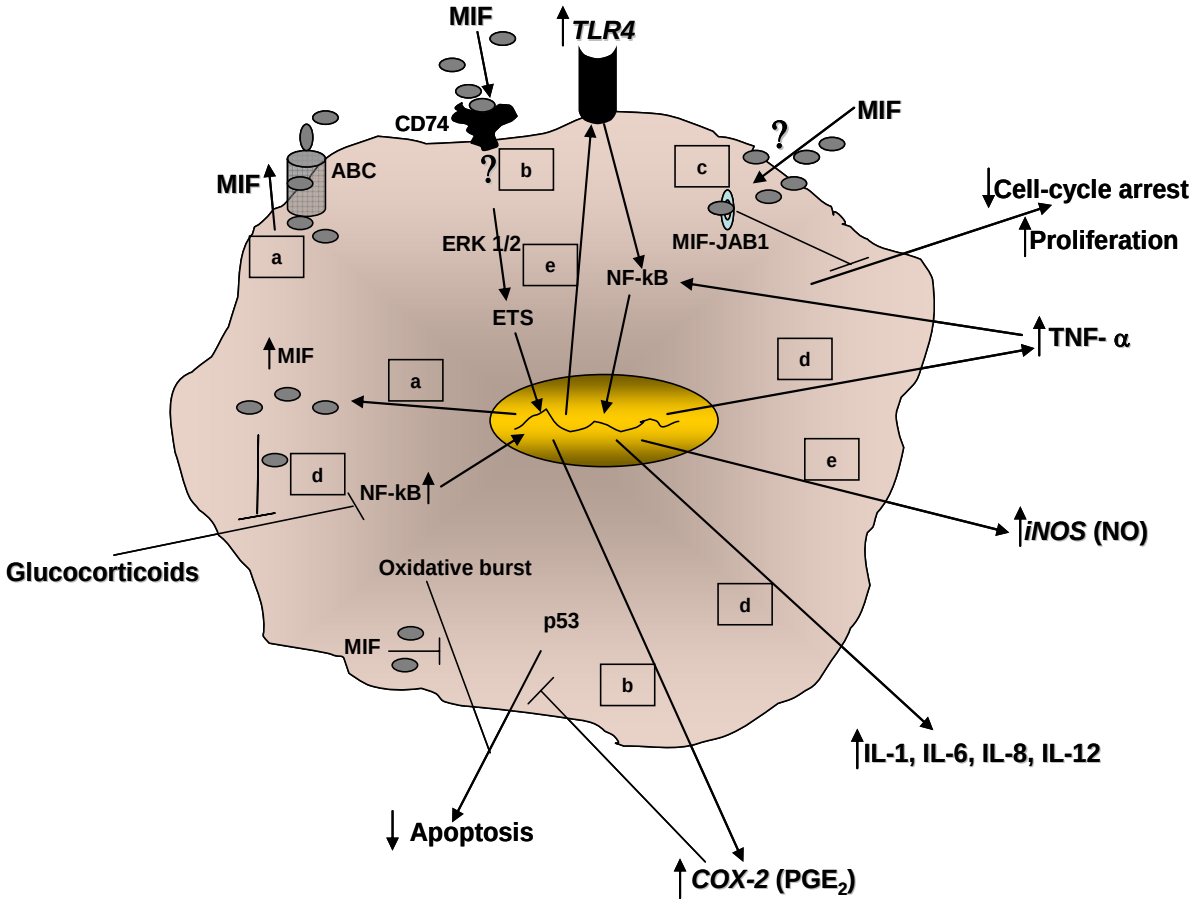


**Figure 3:** ***Biological activities of MIF.***

MIF production is induced by pro-inflammatory stimuli, including lipopolysaccharide (LPS) and IFN-γ. MIF lacks a signal sequence and is believed to be released via a non-classical pathway that involves an ATP-binding cassette (ABC) transporter. b) Although a classical MIF receptor is yet to be identified, binding of MIF to the transmembrane protein CD74 is involved in the activation of the ERK 1/2 pathway that induces COX-2. However, the identities of intracellular adapter proteins involved in transduction of these signals are yet to be determined. COX-2 increases production of $PGE_2$ which mediates MIF-induced suppression of apoptosis by inhibiting p53. MIF also inhibits apoptosis by reducing oxidative burst by an unknown mechanism. c) There is also evidence suggesting for a receptor-independent MIF uptake into the cytoplasm, where it binds with JAB1 to initiate a cascade of events that inhibit cell-cycle arrest and induce increased cell proliferation. d) A unique property of MIF is its ability to reverse glucocortocoid inhibition of NF-kB, thus allowing induction of several pro-inflammatory cytokines

including, IL-1, IL-6, IL-8, Il-12, and TNF-α. Production of TNF-α further augments the pro-inflammatory response by activating NF-κB to induce more mediators including MIF. e) Other biological activities of MIF include induction of inducible NO synthase (iNOS), upregulation of TLR4, and promotion of phagocytosis.

## 1.7 MIF GENETICS

The MIF gene is located on chromosome 22 of the human genome (Donn and Ray 2004). It is a relatively small gene of <1 kb, made up of 205-, 173-, and 183-bp exons separated by 189- and 95-bp introns ((Paralkar and Wistow 1994), Figure 4). This gene is flanked by 250 bp of 3' untranslated region and a 1 kb sequence at the 5' end (Paralkar and Wistow 1994). The MIF promoter lacks a TATA-box, and has one known transcription start site located 97 bp upstream from the initiator methionine (Donn and Ray 2004). Recently, a MIF homologue was identified in *Plasmodium spp*., including *P. falciparum* with about 39% sequence homology with human MIF (Augustijn *et al.* 2007; Cordery *et al.* 2007). In addition, the MIF gene is highly conserved across species, including rats, mice, chickens, parasites, and fishes (Calandra and Roger 2003), suggesting that MIF plays important biological roles across different phylogenies. Five polymorphisms of the MIF gene have been identified to date, including a tetranucleotide short tandem repeat polymorphism (STRP) at -794 ($CATT_{5-8}$), and four single nucleotide polymorphisms (SNPs) at positions -173 (G/C), +24 (A/T), +254 (T/C) and +656 (C/G) (Donn *et al.* 2001; Baugh *et al.* 2002; Donn *et al.* 2002; Renner *et al.* 2005), Figure 4). The +24 SNP is very rare, and has not been well-studied, while the +254 and +656 polymorphisms are positioned in introns and thus not likely to have functional consequences. However, considerable interest has been generated by the polymorphisms in the promoter, since these appear to functionally influence MIF gene expression. The C allele at -173 creates a potential AP-4 binding site, and is generally associated with higher MIF production and increased susceptibility to inflammatory diseases such as arthritis and atopy (Donn *et al.* 2001; Donn *et al.* 2002; Barton *et al.* 2003;

Radstake *et al.* 2005; Renner *et al.* 2005). Similarly, longer CATT repeats at -794 correlate with increased MIF production and higher risk for various types of arthritis including rheumatoid arthritis and juvenile idiopathic arthritis (Baugh *et al.* 2002; Barton *et al.* 2003; Radstake *et al.* 2005). In addition, haplotypes of the two promoter polymorphisms are also associated with MIF production levels, and are better predictors of diseases susceptibility than either polymorphism alone (Donn *et al.* 2002; Barton *et al.* 2003; Donn *et al.* 2004; Hizawa *et al.* 2004).

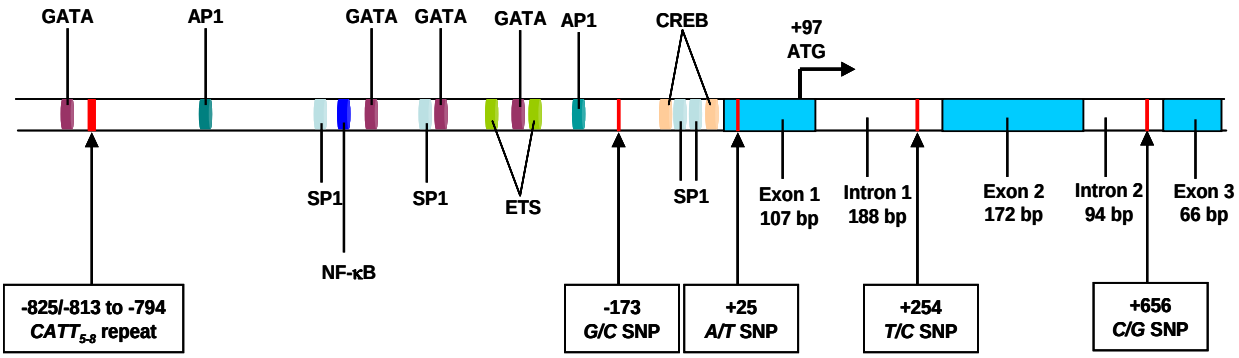


**Figure 4:** ***MIF gene.***

The human MIF gene consists of three exons of 107 bp, 172 bp, and 66 bp, separated by two introns of 188 bp and 94 bp. A single transcription start site, located 97 bp from the methionine codon (ATG), is known. There is ~ 1kb of 5' flanking sequence, containing potential binding sites to several transcription factors including cAMP response element binding protein (CREB), specificity protein 1 (SP1), activator protein 1 (AP1), GATA, E-twenty-six (ETS), and nuclear factor (NF)-κB. Variation at five sites in the MIF gene has been reported: a tetranucleotide short tandem repeat polymorphism at -794 (CATT5-8), and single nucleotide polymorphisms (SNPs) at -173 (G/C), +25 (A/T), +254 (T/C), and +656 (C/G).

## 1.8 ROLE OF MIF IN MALARIAL PATHOGENESIS

Despite the re-emergence of MIF as a central regulator of innate immunity (Calandra and Roger 2003), the role of MIF in malarial pathogenesis remains largely unexplored. Investigations in children with cerebral malaria (CM) showed that MIF levels were expressed at very low levels in blood vessel walls within the brain (Clark *et al.* 2003), and highly expressed in blood vessel walls of peripheral tissue in children with fatal falciparum malaria or sepsis (Clark *et al.* 2003; Clark and Cowden 2003). Additional studies in women with malaria during pregnancy revealed that MIF production was increased in intervillous plasma and in cultured intervillous blood mononuclear cells, but not in peripheral plasma (Chaisavaneeyakorn *et al.* 2002; Chaiyaroj *et al.* 2004; Chaisavaneeyakorn *et al.* 2005). The best evidence for a role for MIF in malarial pathogenesis has come from studies in murine models, where MIF levels were elevated in both peripheral blood and bone marrows of mice with *P. Chabaudi* infections (Martiney *et al.* 2000; McDevitt *et al.* 2006). These investigations also demonstrated that MIF was involved in suppression of erythropoiesis, and circulating levels of MIF correlated with severity of malarial anemia (Martiney *et al.* 2000; McDevitt *et al.* 2006). Interestingly, recent studies have characterized MIF homologs from *Plasmodial* parasites including *P. falciparum* (Augustijn *et al.* 2007; Cordery *et al.* 2007), providing additional evidence for a possible role for MIF in disease pathogenesis. However, studies examining the relationship of MIF production with the pathogenesis of SMA in children that will validate the findings from murine malaria have not been reported. In addition, although there is considerable evidence indicating that variation in immune response genes influences the pathogenesis of severe malaria, the role of genetic

polymorphisms in the MIF promoter in conditioning susceptibility to SMA is unknown. A recent study in a small cohort of Zambian children (n=40) revealed that carriers of >5 CATT repeat alleles at -794 had a higher risk of developing high-density parasitemia (HDP) relative to those with the 5-repeat allele (Zhong *et al.* 2005), demonstrating a potentially important role for MIF polymorphisms in malarial pathogenesis.

# 2.0 CHAPTER TWO: SPECIFIC AIMS

Infections of children with *P. falciparum* lead to increased destruction of RBCs which is complicated by suppression of erythropoiesis and the development of SMA if the infection is not quickly resolved. Although the host-specific factors that regulate these processes are only partially understood, it is clear that the nature and magnitude of innate immune mediator production (McDevitt *et al.* 2004) and genetic variation in host immune response genes (Kwiatkowski 2005) are important determinants of the development and outcomes of severe malaria. Recent studies illustrate that MIF plays a pivotal role in regulating innate immune responses to invading pathogens (reviewed in (Calandra and Roger 2003)). For example, MIF has potent pro-inflammatory properties that protect against *Salmonella typhimurium* (Koebernick *et al.* 2002) and *Leishmania major* (Juttner *et al.* 1998; Xu *et al.* 1998; Satoskar *et al.* 2001). On the other hand, over-production of MIF is associated with enhanced pathogenesis of bacterial sepsis (Bernhagen *et al.* 1993; Bozza *et al.* 1999; Calandra *et al.* 2000), demonstrating that MIF could play both protective and pathogenic roles in different infections. An important property of MIF is its ability to override the immunosuppressive effects of glucocorticoids (Calandra *et al.* 1995), thus allowing the increased production of proinflammatory cytokines, such as IFN-γ and IL-12 (Koebernick *et al.* 2002). In addition, MIF has potent anti-apoptotic properties (Hudson *et al.* 1999; Mitchell *et al.* 2002; Fingerle-Rowson *et al.* 2003) and supports proliferation of embryonic cells (Petrenko *et al.* 2003). Genetic variation in the MIF promoter influences MIF

production and susceptibility to several inflammatory diseases in Cuacasians (Donn *et al.* 2001; Baugh *et al.* 2002; Donn *et al.* 2002; Barton *et al.* 2003; Renner *et al.* 2005), and is associated with HDP in Zambian children with malaria (Zhong *et al.* 2005). The role of genetic variation in the MIF promoter in influencing MIF production and conditioning susceptibility to SMA has not been reported.

Protective immunity to malaria requires an early induction of IFN-γ and IL-12 which drive a robust type 1 immune response for control of parasitemia (Crutcher *et al.* 1995; Stevenson *et al.* 1995; Angulo and Fresno 2002). Therefore, we propose that elevated MIF production during acute malaria protects against SMA by promoting a rapid and potent innate immune response that could result in more efficient control over the initial phases of parasitemia. In addition, since phagocytosis of Hz by monocytes and neutrophils is associated with severe malaria (Nguyen *et al.* 1995; Luty *et al.* 2000; Lyke *et al.* 2003; Casals-Pascual *et al.* 2006) and dysregulation in cytokine production (Pichyangkul *et al.* 1994; Sherry *et al.* 1995; Perkins *et al.* 2003; Keller *et al.* 2004a; Keller *et al.* 2004b; Ochiel *et al.* 2005; Keller *et al.* 2006a; Keller *et al.* 2006b), we postulate that increased deposition of Hz in phagocytes will enhance SMA pathogenesis by suppressing MIF production. Furthermore, since MIF supports cell proliferation, adequate MIF levels will be required for survival and efficient proliferation of erythroid progenitors to replace lost RBCs and prevent anemia during *P. falciparum* infection. In addition, we postulate that MIF promoter variants determine MIF production at baseline and in response to malaria infection, thereby influencing susceptibility to SMA.

## 2.1 SPECIFIC AIM 1

**To determine the relationship of peripheral blood MIF production with circulating levels of MIF-associated inflammatory mediators, and to investigate the role of MIF in the development of severe anemia in children with acute *P. falciparum* infection.**

***Hypothesis 1:*** *Peripheral blood MIF production correlates with induction of pro-inflammatory mediators including IFN-γ, IL-12, TNF-α, and NO in children with acute malaria.*

***Hypothesis 2****: Elevated basal MIF production is protective against the development of severe malaria during subsequent P. falciparum infections*

Circulating MIF protein and PBMC MIF transcripts levels were determined in children with acute malaria and healthy controls using ELISA and real time RT-PCR respectively. In addition, circulating levels of the pro-inflammatory mediators IFN-γ, IL-12, TNF-α, $PGE_2$, and NO, and the anti-inflammatory cytokines IL-10 and TGF-β1 were examined. Multivariate linear regression analyses were performed to determine the relationship between MIF and the other mediators. Furthermore, using longitudinal data available for the cohort, MIF levels in healthy children with a history of mild malaria were compared to those with a history of severe malaria to investigate the role of basal MIF production and disease susceptibility.

Additional studies were conducted to investigate the following hypotheses:

***Hypothesis 3:*** *Peripheral blood MIF production is associated with anemia severity in children with acute P. falciparum malaria*

***Hypothesis 4**: Phagocyte acquisition of malarial pigment (hemozoin) leads to suppression of MIF production during acute malaria*

Children were recruited from Siaya District in western Kenya, where *P. falciparum* transmission is holoendemic, and the primary clinical manifestations of severe malaria are severe anemia and HDP (McElroy *et al.* 2000; Ong'echa *et al.* 2006). Peripheral blood was obtained from children with differing severities of malarial anemia or uncomplicated malaria, and healthy aparasitemic controls and used to examine circulating MIF levels, production of MIF in cultured PBMC, and PBMC MIF transcript levels. In addition, a complete hematological evaluation of study participants was conducted, including full blood counts, reticulocyte counts, parasitemia and Hb determination, and quantification of pigment-containing monocytes (PCM) and pigment-containing neutrophils (PCN). The relationship between MIF production and anemia severity was examined by comparing MIF levels across groups, and also through a multivariate regression model with Hb as the dependent variable, and controlling for the confounding effects of age, gender, and parasitemia. Furthermore, given the *in vivo* role of Hz in cytokine dysregulation (Luty *et al.* 2000; Perkins *et al.* 2003; Keller *et al.* 2006b) and pathogenesis of SMA (Wickramasinghe and Abdalla 2000; Casals-Pascual *et al.* 2006), the relationship of PCN and PCM with anemia severity and MIF production was examined. Additional experiments investigated the effects of purified *P. falciparum* Hz (pfHz) on MIF production in cultured malaria-naïve PBMC and purified monocytes.

## 2.2 SPECIFIC AIM 2

**To investigate the role of MIF in proliferation and differentiation of erythroid progenitor cells.**

*Hypothesis 1: Endogenous MIF production is required to promote efficient proliferation of erythroid progenitors.*

*Hypothesis 2: MIF-regulated inflammatory mediators TNF-α and NO are more detrimental to erythroid progenitor survival and differentiation than MIF.*

Given the difficulties in obtaining sufficient quantities of erythroid progenitors from bone marrows of severely anemic children and the lack of a reliable *ex vivo* or *in vitro* model for examining erythroid development, one of the aims of this study was to development a simple and reliable model to study erythropoiesis *in vitro*. Based on recently demonstrated methods for cultivating CD34+ hematopoietic stem cells in liquid culture and inducing them towards erythroid differentiation (Freyssinier *et al.* 1999; Neildez-Nguyen *et al.* 2002), an *in vitro* model of erythropoiesis was developed and used to evaluate the effects of MIF and MIF-regulated mediators, NO and TNF-α, on erythropoiesis. For these investigations, peripheral blood CD34+ stem cells were induced to develop into RBCs in liquid culture by stimulation with erythropoietin (Epo) in the presence of anti-MIF blocking antibodies, recombinant human (rh) MIF, rhTNF-α or NO donor-compounds. Erythroid cell proliferation was measured by a methylthiazoletetrazolium (MTT)-based assay, apoptosis by detection of nucleosomes using

ELISA, and maturation of erythroid cells by assessing surface expression of CD34, CD45, CD71 and glycophorin-A (GPA) using flow cytometry.

## 2.3 SPECIFIC AIM 3

**To examine the influence of genetic variation at positions -173(G/C) and -794 ($CATT_{5-8}$) of the MIF promoter on MIF production and susceptibility to SMA in children.**

***Hypothesis 1:*** *MIF -173 and -794 variants that are associated with decreased MIF production also condition increased susceptibility to SMA*

***Hypothesis 2:*** *Haplotypes of the MIF -173 and -794 polymorphisms are stronger predictors of SMA susceptibility and MIF production levels than either polymorphism alone*

To test these hypotheses, DNA samples obtained from the Kenyan children enrolled for *Specific Aim 1* were analyzed for MIF -173 and -794 genotypes. The -173 SNP was examined in a high-throughput manner using allelic discrimination by real time RT-PCR, and results were validated by testing 10% of the samples using conventional restriction fragment length polymorphism (RFLP)-PCR. The -794 STRP was analyzed by PCR using florescent-labeled primers and products were resolved by capillary electrophoresis. The relationships of MIF -173 and -794 genotypes and haplotypes with SMA were examined using multivariate logistic regression models that controlled for the confounding effects of age, gender, sickle cell trait and HIV-1 status. In addition, data obtained in *Specific Aim1* on circulating MIF levels and PBMC

MIF production were analyzed according to genotypic and haplotypic groups in parasitemic and aparasitemic groups to determine the functional association between genotypic variants and MIF production.

# 3.0 CHAPTER THREE: RESULTS, SPECIFIC AIM 1

## 3.1 HYPOSTHESIS 1: PRESENTATION OF MANUSCRIPT ENTITLED: *DECREASED CIRCULATING MACROPHAGE MIGRATION INHIBITORY FACTOR (MIF) PROTEIN AND BLOOD MONONUCLEAR CELL MIF TRANSCRIPTS IN CHILDREN WITH PLASMODIUM FALCIPARUM MALARIA*

*Hypothesis 1: Peripheral blood MIF production correlates with induction of pro-inflammatory mediators including IFN-γ, IL-12, TNF-α, and NO in children with acute malaria.*

To address this hypothesis, circulating levels of MIF, IFN-γ, TNF-α, IL-12, IL-10, TGF-β1, and $PGE_2$, PBMC nitric oxide synthase (NOS) activity, and PBMC MIF transcripts levels were examined in a group of Gabonese children presenting with acute malaria and healthy age-matched aparasitemic children. The relationship of MIF levels with malaria disease and production of other inflammatory mediators was investigated. The results of these investigations are presented in the following manuscript published in the journal *Clinical Immunology* (Clin. Immunol. 2006, 119: 219-225; reproduced with permission from Elsevier Limited).

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### 3.1.1 Footnote page

Part of this work was presented at the 53nd Annual Meeting of the American Society of Tropical Medicine and Hygiene (ASTMH, Abstract # 775) held in Miami Beach, Florida USA (7th-11th November 2004). The study was approved by the Ethics Committee of the International Foundation of the Albert Schweitzer Hospital in Lambarènè, Duke University Medical Center Investigational Review Board, and the University of Pittsburgh Investigational Review Board, and informed consent was obtained from all participants or the parents of participating children.

There is no conflict of interest for any of the authors of the manuscript due to either commercial or other affiliations.

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### 3.1.2 Abstract

*Plasmodium falciparum* malaria remains one of the most frequently lethal diseases affecting children in sub-Saharan Africa, yet the immune mediators that regulate pathogenesis are only partially defined. Since macrophage migration inhibitory factor (MIF) is important for regulating innate immunity in bacterial and parasitic infections, circulating MIF and peripheral blood mononuclear cell (PBMC) MIF transcripts were investigated in children with acute falciparum malaria. Peripheral blood levels of MIF-regulatory cytokines and effector molecules, including interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-12, IL-10, transforming growth factor (TGF)-β1, bicyclo-prostaglandin (PG) $E_2$, and nitric oxide synthase activity were also determined. Circulating MIF and PBMC MIF mRNA were significantly lower in children with acute malaria relative to healthy, malaria-exposed children. Peripheral blood MIF levels showed no association with either parasitemia or hemoglobin concentrations. Circulating MIF was, however, significantly associated with IL-12 and TGF-β1. Multiple regression analyses revealed that IFN-γ was the most significant predictor of peripheral blood MIF concentrations. These findings suggest that reduced MIF production may promote enhanced disease severity in children with falciparum malaria.

Running title: Low peripheral blood MIF in children with malaria

Keywords: Macrophage migration inhibitory factor (MIF); *Plasmodium falciparum*; malaria; anemia; immunity; cytokines.

### 3.1.3 Introduction

Malaria is a major global health problem with 300 to 500 million clinical cases each year resulting in more than 2 million deaths (WHO 2000), with the majority of malaria-associated mortality occurring in African children less than 5 years of age (Guilbert 2003). Although four species of the malaria parasite infect humans, malaria-related mortality is primarily due to *Plasmodium falciparum* (WHO 2000). The clinical presentation of falciparum malaria in children varies from asymptomatic infection to severe life-threatening complications such as one or more of the following: hyperparasitemia, hypoglycemia, cerebral malaria, severe anemia, respiratory distress, hyperlactatemia or others (WHO 2000). Although the factors that determine disease severity during acute malaria are not clearly defined, host genetic factors, age of first exposure, and rate of exposure (endemicity) are all important for conditioning the acquisition of naturally acquired immunity.

One cytokine that is largely unexplored in human malaria is macrophage migration inhibitory factor (MIF). Recent studies illustrate that MIF is a critical regulatory factor of the innate immune response in that MIF can promote both protective immunity and enhanced pathogenesis in bacterial and parasitic infections (Bernhagen *et al.* 1993; Calandra *et al.* 1998; Juttner *et al.* 1998; Xu *et al.* 1998; Bozza *et al.* 1999; Calandra *et al.* 2000; Satoskar *et al.* 2001; Koebernick *et al.* 2002; Rodriguez-Sosa *et al.* 2003). Although MIF was initially identified for its role in delayed-type hypersensitivity and thought to be primarily T-cell-derived (Bloom and Bennett 1966; David 1966), additional research has shown that MIF is a pro-inflammatory mediator released by the anterior pituitary gland in response to endotoxins (Bernhagen *et al.* 1993), and by cells of the monocyte/macrophage lineage in response to bacterial toxins and pro-

inflammatory cytokines, such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α (Calandra *et al.* 1994). Consistent with the fact that pro-inflammatory-derived cytokines induce MIF expression, anti-inflammatory cytokines, such as IL-10 down-regulate MIF (Wu *et al.* 1993).

Previous studies in a murine model of malaria showed that infection with *P. chabaudi* increased MIF in circulation, which was associated with enhanced anemia (Martiney *et al.* 2000), suggesting that increased MIF production may be an important factor for promoting malarial anemia (MA) (Martiney *et al.* 2000). Recent investigations in children with cerebral malaria (CM) showed that MIF levels were expressed at very low levels in blood vessel walls within the brain (Clark *et al.* 2003), and highly expressed in blood vessel walls of peripheral tissue in children with fatal falciparum malaria or sepsis (Clark *et al.* 2003; Clark and Cowden 2003). Additional studies in women with malaria during pregnancy revealed that MIF production was increased in intervillous plasma and in cultured intervillous blood mononuclear cells, but not in peripheral plasma (Chaisavaneeyakorn *et al.* 2002; Chaiyaroj *et al.* 2004; Chaisavaneeyakorn *et al.* 2005). While these earlier studies in rodent and human malaria suggest that MIF may be pathogenic by promoting an excessive pro-inflammatory response, adequate MIF levels may also be necessary to allow a potent inflammatory response that could provide protection against severe disease by ensuring rapid and efficient parasite clearance.

To our knowledge, the role of MIF in regulating the immunological response to acute non-cerebral *falciparum* malaria in children has not previously been examined. In the current study, peripheral blood levels of MIF and peripheral blood mononuclear cell (PBMC) MIF transcripts were determined in children with acute *P. falciparum* malaria in which the primary manifestation of acute malaria was hyperparasitemia and anemia. In addition, the association

between MIF and other immune mediators, such as IFN-γ, TNF-α, IL-12, IL-10, TGF-β, bicyclo-$PGE_2$ and NO was investigated.

### 3.1.4 Materials and methods

#### 3.1.4.1 *Study participants.*

Children (ages 2-8 years) were selected from a longitudinal prospective study at the Albert Schweitzer Hospital in Lambarènè, Gabon (equatorial West Africa), an area of hyperendemic malaria transmission where hyperparasitemia and anemia are the predominant complications of acute malaria (Kun *et al.* 1998; Kun *et al.* 1998; Perkins *et al.* 1999). Upon enrollment in the study, finger-prick blood (<100 μL) was obtained to determine parasitemia and anemia status. Peripheral blood smears were prepared and stained with Giemsa reagent and examined under oil immersion for malaria parasites. Asexual malaria parasites were counted using the Lambarènè method (Planche *et al.* 2001). Determinations of Hb levels were performed on finger-prick blood using a HemoCue system (HemoCue AB, Angelholm, Sweden). Children presenting with acute *P. falciparum* malaria (Mal, n=23) were treated promptly with the appropriate antimalarials and supportive therapy. None of the children in the current study had cerebral malaria. Children diagnosed with any diseases other than malaria were excluded from the study. Healthy aparasitemic controls (AC, n=25) were children in the convalescent phase of malaria and were currently free of malaria parasites and any other detectable disease for one or more months.

Participation in the study was completely voluntary and written informed consent was obtained from the parent/guardian of the pediatric participants. The study received approval from the ethics committee of the International Foundation of the Albert Schweitzer Hospital, Duke University Medical Center Investigational Review Board, and the University of Pittsburgh Institutional Review Board.

**3.1.4.2** ***Sample collection.***

Before delivery of anti-malarials and/or any other treatment interventions, venous blood was drawn into heparinized tubes (2-3mL), centrifuged and the plasma removed and stored frozen at -70$^{o}$C until analysis. PBMCs were isolated using Ficoll-Hypaque as described previously and stored frozen at -70$^{o}$C until the time of use (Weinberg *et al.* 1981).

**3.1.4.3** ***Nitric oxide synthase (NOS) enzyme activity.***

Cell lysates were prepared from frozen PBMCs and NOS enzyme activity was determined by measuring the conversion of L-[$^{14}$C]arginine to L-[$^{14}$C]citrulline (Weinberg *et al.* 1994). Samples were assayed in triplicate and the average NOS enzyme activity was expressed as picomoles of citrulline/mg of protein. This method of assaying NO production was selected since circulating markers of NO production, such as nitrate, can be influenced by dietary intake of nitrates.

**3.1.4.4** ***Cytokine and Prostaglandin measurements.***

Cytokine levels in plasma were determined using quantitative sandwich ELISA. To eliminate variation between assays, all samples were assayed at the same time for each of the cytokines and effector molecules. MIF was measured using a matched antibody pair from R & D systems (cat no. MAB289 and BAF289), according to the manufacturer's recommendations. Similarly, concentrations of IFN-γ, TNF-α, TGF-β1, and IL-10 were determined using commercially available reagents (R & D systems, Minneapolis). For IL-12 measurements, a monoclonal antibody specific for the active heterodimer of IL-12 was used. To exclude TGF-β1 released from platelets, a separate aliquot of platelet-poor plasma was generated by centrifugation at 10,000g for 15 minutes. The resulting plasma samples were activated by incubation with 2.5 N

acetic acid/10N urea for 10 minutes, followed by neutralization with 2.7 N NaOH/1 M HEPES, before determination of TGF-β1. All samples were assayed in duplicate and the sensitivities of detection were as follows: MIF >62.5 pg/mL; IFN-γ >3.0pg/mL; TNF-α >4.4 pg/mL; IL-12 >5.0 pg/mL; IL-10 >3.0 pg/mL; and TGF-β1 >7.0 pg/mL). $PGE_2$ and the intermediary metabolite were converted to the stable form, bicyclo-$PGE_2$, and measured using a commercially available EIA (Cayman Chemical, San Diego), according to our previously described methods (Perkins *et al.* 2001).

**3.1.4.5** ***MIF mRNA analyses.***

Total RNA was extracted from PBMCs using the GITC method as described previously (Chomczynski and Sacchi 1987). The RNA yield was assessed by spectrophometry and 1μg of total RNA transcribed into cDNA using MuLV reverse transcriptase (Applied Biosystems, Foster City, CA). A MIF specific fluorogenic PCR primer/probe set (assay ID Hs00236988_g1, Applied Biosystems, Foster City, CA) was used for real time PCR run on the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). Each sample was run in duplicate. The housekeeping gene, β-actin (assay ID NM_001101, Applied Biosystems, Foster City, CA), was amplified from each sample to normalize template concentrations. Comparisons between samples were made using differences in critical threshold values ($\Delta C_T$ = critical threshold cycle of MIF – critical threshold cycle for β-actin). MIF mRNA levels in PBMCs of children were expressed as fold change relative to β-actin mRNA ($2^{-\Delta CT}$).

#### 3.1.4.6 *Statistical analyses.*

Non-parametric data are presented as box plots showing medians and interquartile range, while normalized data are presented as bar graphs showing means and standard errors of means (SEM). Age, Hb concentration, body temperature and plasma levels of cytokines and effector molecules between groups were compared using Mann-Whitney U-tests. MIF transcript levels between groups of children were normalized against β-actin levels and compared by Student's *t* test. Linear associations between MIF and cytokines and effector molecules were examined via Pearson correlations and multiple regression analysis. Statistical significance for all tests was set at $P<0.05$.

### 3.1.5 Results

#### 3.1.5.1 *Patient Characteristics.*

Children with acute *P. falciparum* malaria (Mal, n=23) were recruited from the Albert Schweitzer Hospital in Lambarènè, Gabon. Aparasitemic, age-matched controls (AC, n=25) were recruited from a longitudinal cohort study which allowed for a known history of falciparum malaria in children from the same geographic location. The clinical, parasitological, and laboratory characteristics of the AC and Mal groups are summarized in Table 1. There were no significant differences in age (mos) between the two groups ($P$=0.2). Hb concentrations (g/dL) were significantly higher in the control group ($P$<0.01), while body temperature (◦C) was significantly higher in children with acute malaria ($P$<0.05).

#### 3.1.5.2 *Plasma MIF in children with acute malaria.*

To investigate the role of MIF in the pathogenesis of childhood MA, plasma MIF levels were determined in children with acute *P. falciparum* malaria and age-matched aparasitemic controls. Since erythrocytes contain large quantities of MIF (Mizue *et al.* 2000), plasma samples were used instead of serum to avoid the possible influence of MIF released from lysis of erythrocytes that can result during the clotting process. Compared to aparasitemic controls, children with acute malaria had significantly lower plasma MIF concentrations ($P$<0.05, Figure 5A). The mean (range) of MIF levels were 1224 (190-3893) for children with acute malaria versus 1627 (710-3829) for aparasitemic controls.

#### 3.1.5.3 *MIF mRNA in PBMCs during acute malaria.*

Determining the cellular source responsible for altered MIF levels in circulation can be complex because MIF is produced from a number of different sources including cells of the endocrine system and several immune cells (Bernhagen *et al.* 1993; Calandra *et al.* 1994; Calandra and Roger 2003). Since we have previously shown that reductions of circulating cytokines and effector molecules is due to altered PBMC transcriptional regulation in children with acute malaria (Perkins *et al.* 2001; Ochiel *et al.* 2005), PBMC MIF transcripts were examined by real time RT-PCR. There was a positive, but non-statistically significant association between circulating MIF protein and PBMC transcript levels (r=0.26, $P$=0.156). MIF transcripts were significantly lower in children with acute malaria compared to aparasitemic controls ($P$<0.05, Figure 5B), suggesting that reductions in circulating MIF may be, at least in part, related to reduced MIF mRNA expression in PBMC.

#### 3.1.5.4 *Production of MIF-associated cytokines and mediators in children with acute malaria.*

To examine the association between altered MIF and other important immunological mediators during acute falciparum malaria, levels of IFN-γ, TNF-α, IL-12, TGF-β1, IL-10, bicyclo-$PGE_2$, and NOS enzyme activity were determined. Circulating levels of inflammatory mediators in aparasitemic children and children with acute malaria are shown in Table 2. Children with acute malaria had significantly higher levels of IFN-γ ($P$<0.01), TNF-α ($P$<0.005), IL-10 ($P$<0.0001), and NOS enzyme activity ($P$<0.005). In contrast, bicyclo-$PGE_2$ and TGF-β1 were significantly reduced in children with acute malaria ($P$<0.01 and $P$<0.001, respectively), while IL-12 was non-significantly reduced in children with malaria ($P$=0.5). Portions of the cytokine and effector molecule data were previously reported as part of our studies examining acute falciparum

malaria in Gabonese children (Perkins *et al.* 1999; Luty *et al.* 2000; Perkins *et al.* 2000; Perkins *et al.* 2001; Keller *et al.* 2004b).

**3.1.5.5** ***Associations between cytokines and effector molecules and MIF in children with acute malaria.***

To examine the strength and direction of linear association between plasma MIF and the various cytokines and effector molecules, Pearson correlations and a multiple regression analysis were conducted. For these analyses, MIF was logarithmically transformed, and several of the cytokines were either logarithmically or square-root transformed, to reduce positive skewness and increase normality. There was a significant positive correlation between MIF and both IL-12 ($r$=0.22, $P$<0.05) and TGF-β1 ($r$=0.27, $P$<0.01). When the study participants were stratified into two groups according to the case definitions (i.e., AC and Mal), the association between IL-12 and MIF remained significant for children with acute malaria ($r$=0.37, $P$<0.05), but was non-significant for the aparasitemic controls ($r$=0.21, $P$=0.2, Figure 6A). The association between TGF-β1 and MIF was also reduced to non-significance when the two groups were examined separately ($r$=0.19, $P$=0.2, for Mal; $r$=0.30, $P$=0.08, for AC; Figure 6B).

In the multivariate analysis, both aparasitemic controls and children with acute malaria were included in the model. For these analyses, MIF served as the criterion variable; the predictors were age, parasitemia, cytokines (i.e., IFN-γ, TNF-α, IL-12, IL-10, and TGF-β), and effector molecules (i.e., bicyclo-$PGE_2$ and NOS enzyme activity), and the covariate consisted of group status (Mal versus AC). The regression was hierarchical with group status entered first in the model and all of the predictors entered in the second step. The multivariate model revealed that IFN-γ was the most significant predictor of MIF, with a standardized partial regression

coefficient (β-weight) of 0.31 ($P$=0.05). Moreover, the positive association was independent of disease severity since group status was controlled for in the model, illustrating that IFN-γ is an important cytokine for regulating peripheral blood MIF levels in both aparasitemic, malaria-exposed children and children with acute malaria.

### 3.1.6 Discussion

To our knowledge, this is the first published report on MIF production and regulation in children with non-cerebral malaria. Consistent with present results showing that circulating MIF levels are decreased in children with malaria, previous studies illustrated that plasma MIF protein was decreased (although not statistically significant) in women with *P. falciparum* malaria during pregnancy (Chaisavaneeyakorn *et al.* 2002; Chaiyaroj *et al.* 2004). However, it is interesting to note that intervillous plasma levels in these same women were increased during acute malaria (Chaisavaneeyakorn *et al.* 2002; Chaiyaroj *et al.* 2004). Plasma MIF levels reported here (≈ 1-4 ng/mL) for aparasitemic controls are slightly lower than those reported elsewhere in adult Caucasians (Calandra *et al.* 2000), but are within the range of levels in peripheral plasma of malaria-exposed African women (Chaisavaneeyakorn *et al.* 2002; Chaiyaroj *et al.* 2004). Post-mortem histochemistry studies in Malawian children with CM illustrated that MIF protein expression is largely absent within cerebral structures, but highly expressed in chest wall blood vessels (Clark *et al.* 2003). Since MIF has recently been shown to be regulated differently in individual compartments of malaria-infected placentas (Chaisavaneeyakorn *et al.* 2002; Chaiyaroj *et al.* 2004; Chaisavaneeyakorn *et al.* 2005), it is feasible that peripheral blood MIF regulation may differ from that in central and peripheral tissues, thus accounting for the differences in MIF responses observed here versus those in placental malaria and CM.

Findings presented here illustrate that MIF transcript levels in *ex vivo* PBMCs are decreased in acute malaria relative to aparasitemic controls, and that reduced MIF gene expression corresponds with decreased plasma MIF concentrations. These results suggest that PBMC may be an important source of circulating MIF during acute malaria. Decreased MIF

transcription in PBMC from children with acute malaria could be due to parasite-derived products such as hemozoin, and/or parasite-induced changes in host immune mediators, such as cytokines and effector molecules. In the current study, MIF was not significantly associated with parasitemia (data not shown), suggesting that an altered inflammatory milieu rather than parasitemia per se may account for suppression of MIF in acute disease.

Infection of mice with *P. chabaudi* resulted in increased serum MIF levels that were associated with enhanced levels of anemia (Martiney *et al.* 2000). Results presented here show that MIF levels declined in children with malarial anemia compared to healthy, malaria-exposed children. However, MIF levels were not significantly associated with Hb concentrations (data not presented). Since glucocorticoids regulate MIF production differently in the hypothalamo-pituitary-adrenal axis of humans versus rodents (Isidori *et al.* 2002), it remains to be determined if murine and human regulation of MIF production differ during a malaria infection.

A number of studies have identified IL-12 as a central factor for promoting a protective immune response in animal models of malaria (reviewed in (Crutcher *et al.* 1995)). In addition, we have shown that low IL-12 levels are associated with increased disease severity in children with malaria (Luty *et al.* 2000; Perkins *et al.* 2000). The association between MIF and IL-12 levels reported here suggests a potentially significant biologic relationship in which reduced MIF levels combined with low IL-12 could enhance malaria disease severity. Previous studies have shown that induction of other proinflammatory mediators (TNF-$\alpha$, NO and $PGE_2$) are related to MIF production (Calandra *et al.* 1994; Juttner *et al.* 1998; Koebernick *et al.* 2002; Mitchell *et al.* 2002). In the present studies, we found no evidence to suggest that MIF may be responsible for increased TNF-$\alpha$ and NO production during acute malaria, as MIF levels were decreased in children with malaria in the presence of increased TNF-$\alpha$ and NO. On the contrary, increased

NO production could be contributing to suppression of MIF, since NO has potent immunosuppressive properties. Furthermore, although both MIF and $PGE_2$ were decreased in children with acute malaria, the correlation between MIF and $PGE_2$ was not significant. Earlier studies in Gabonese children demonstrated that potent IFN-γ responses in PBMC stimulated with malarial antigens were associated with protection against severe malaria (Luty *et al.* 1999). Therefore, the finding that IFN-γ had the greatest predictive value on circulating MIF levels raises the possibility that IFN-γ be an important factor for regulating MIF production during acute malaria. This potentially important relationship requires additional studies for this postulate to be confirmed.

Increased levels of IL-10 suppress MIF transcription in both human and murine monocytes/macrophages (Wu *et al.* 1993). In this study, circulating IL-10 was highly elevated in children with acute malaria. Although elevated IL-10 may contribute to decreased MIF protein and transcripts, the statistically non-significant correlation between MIF and IL-10 levels does not support the notion that over-production of IL-10 is responsible for down-regulation of MIF gene expression.

Experiments in murine models of malaria illustrate that TGF-β is an important molecule for regulating disease severity (Omer and Riley 1998; Tsutsui and Kamiyama 1999). We have further shown that reduced levels of TGF-β1 are associated with enhanced disease severity in children with acute malaria (Luty *et al.* 2000; Perkins *et al.* 2000). Thus, it is possible that concomitantly reduced MIF, IL-12 and TGF-β1 levels in children with acute malaria may promote an ineffective immune response that culminates in enhanced pathogenesis. It is important to note that interrelationships between MIF and other immune mediators described in this study are not conclusive evidence of cross-regulation among these mediators during acute

malaria. Therefore, additional mechanism-based studies aimed at defining the role of MIF in regulating cytokine responses during malaria may offer important insight into the role of innate immunity in determining malaria disease outcomes.

### 3.1.7 Acknowledgements

We thank the following staff members of Albert Schweitzer Hospital in Lambaréné Gabon for technical assistance to the study: Dr. Anita van den Biggerlaar, Judith Jans, Dr. Hanna Knoop, Dr. Doris Luckner, Barbara Moritz, Anselme Ndzengue, Marcel Nkeyi, Dr. Daniela Schmid, and Dr. Milena Sovric.

The study was conducted at the University of Pittsburgh, and supported in part by the National Institutes of Health Grant (AI-51305-01 DJP) and (AI-41764 JBW), the VA Research Service (JBW) and Fogarty International Training Grant (5D43-TW00588-4 DJP).

**Table 1:** ***Clinical, parasitological, and laboratory characteristics of study participants.***

| Characteristic | Aparasitemic controls | Acute malaria |
|---|---|---|
| Number of children, *n* | 25 | 23 |
| Age, *years* | 4.6 (0.6) | 4.9 (0.3) |
| Hyperparasitemia* | | |
| ≥ 200,000 parasites/μL | 0 | 10 |
| Severe anemia* | | |
| Hemoglobin ≤6.0 g/dL | 0 | 2 |
| PCV ≤ 20% | 0 | 7 |
| Parasitemia | | |
| *Parasites/μL* | 0 | 160246 (30432) |
| *Geometric mean* | 0 | 90591.5 |
| Hemoglobin, *g/dL* | 11.6 (0.5) | 9.0 (0.4) |
| Platelet count, $X\ 10^6/L$ | ND | 170.0 (18.0) |
| Glucose, *mg/dL* | ND | 85.0 (4.3) |
| Body temperature, $^oC$ | 37.1 (0.2) | 38.8 (0.2) |

Data are presented as mean (SEM) or geometric mean for parasitemia.

* indicates number of children in each category.

ND, not determined.

Differences between the two groups were statistically significant for hemoglobin ($P<0.01$) and body temperature ($P<0.05$), but not age ($P=0.2$) (Mann-Whitney U-test).

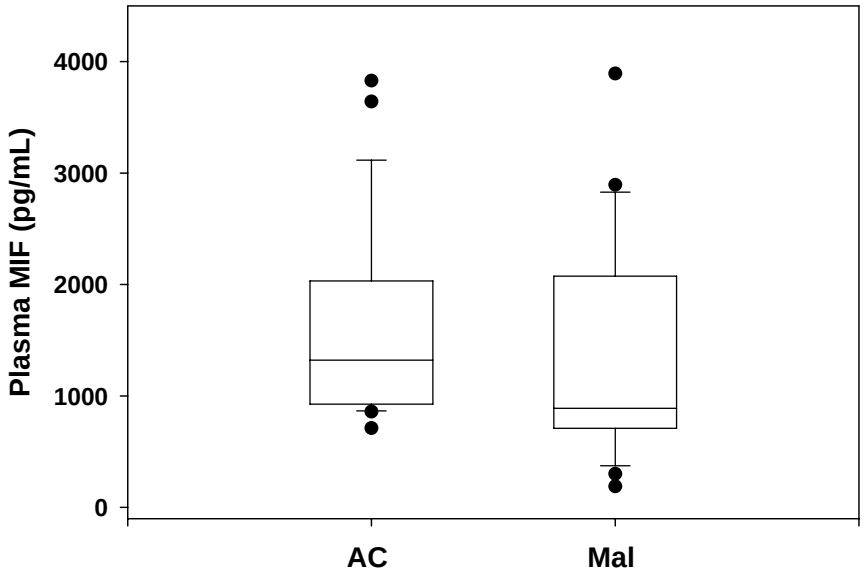
**Table 2:** ***Levels of cytokines and effector molecules in children with acute malaria compared to aparasitemic controls.***

| Cytokines and Effector Molecules | Aparasitemic controls | Acute malaria | *p*-value |
|---|---|---|---|
| MIF (pg/mL) | 1493 (*936-2428*) | 913 (*710-2252*) | < 0.05 |
| IL-12 (pg/mL) | 40 (*29-77*) | 61 (*2-82*) | = 0.5 |
| IFN-γ (pg/mL) | 20 (*0-27*) | 36 (*16-59*) | < 0.01 |
| TNF-α (pg/mL) | 64 (*30-118*) | 152 (*89-279*) | < 0.005 |
| NOS (pmol/mg protein) | 232 (*159-372*) | 522 (*328-611*) | < 0.005 |
| Bicyclo-$PGE_2$ (pg/mL) | 29 (*21-38*) | 19 (*9-26*) | < 0.01 |
| IL-10 (pg/mL) | 0 (*0-18*) | 152 (*99-268*) | < 0.0001 |
| TGF-β1 (ng/mL) | 16 (*11-20*) | 8 (*6-11*) | < 0. 001 |

Data are presented as median (interquartile range).

Levels of cytokines and effector molecules between the two groups were compared using Mann-Whitney U-test, and $p < 0.05$ was considered statistically significant.

**A.**




**B.**

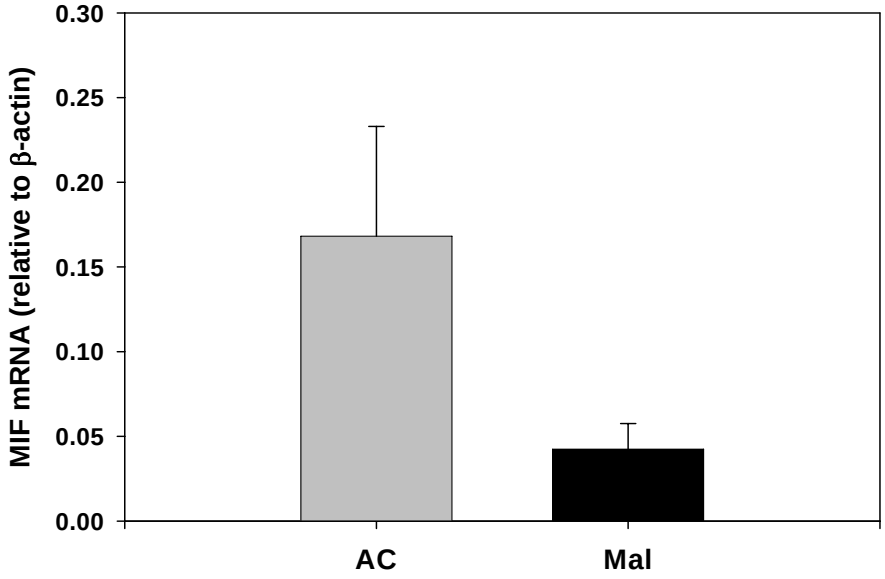
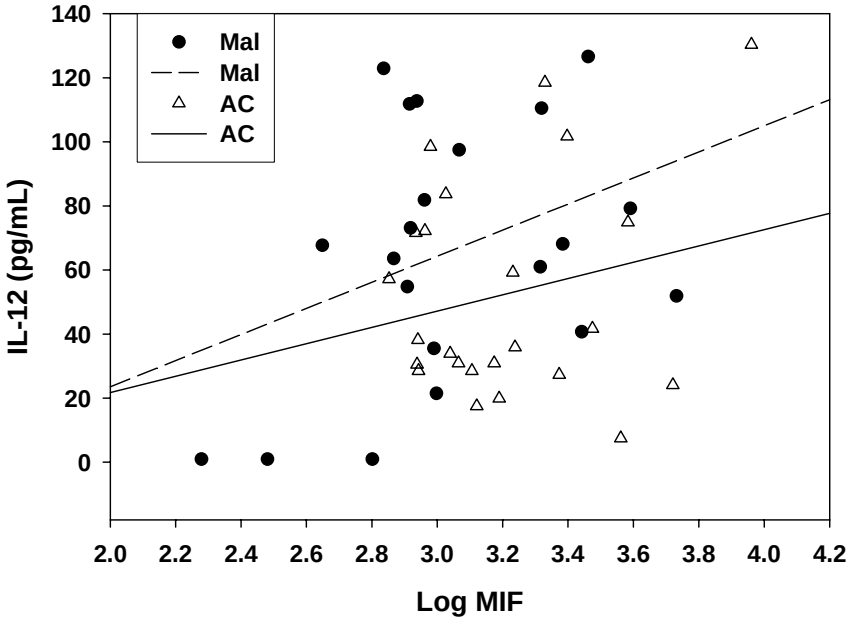



**Figure 5:** ***Circulating MIF protein and PBMC MIF transcripts in children with acute P. falciparum malaria.***

**Figure 5:** ***Circulating MIF protein and PBMC MIF transcripts in children with acute P. falciparum malaria.*** (A) Plasma levels of MIF in children with acute malaria (Mal, n=23) and aparasitemic controls (AC, n=25) were measured by ELISA. Data are presented as box plots where the box represents the interquartile range, the line through the box is the median, whiskers indicate the $10^{th}$ and $90^{th}$ percentiles and individual symbols are outliers. Differences in MIF levels between the groups were statistically significant ($P<0.05$, Mann-Whitney U-test). (B) MIF-specific mRNA levels in PBMC of children with Mal (n=19) and AC (n=20) were determined by real time RT-PCR and normalized against β-actin mRNA levels. Bars represent mean $\pm$ SEM of MIF mRNA expressed relative to β-actin transcripts for the two groups. Differences in MIF transcript levels were statistically significant ($P<0.05$, Student's t-test).

**A.**




**B.**

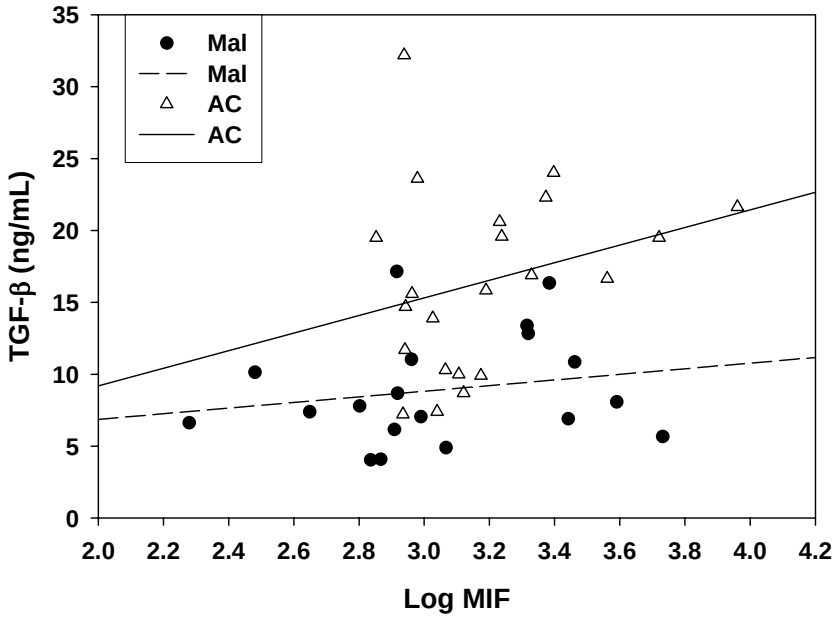



**Figure 6:** ***Association of plasma MIF with IL-12 and TGF-β in children with acute malaria and aparasitemic controls.***

***Figure 6: Association of plasma MIF with IL-12 and TGF-β in children with acute malaria and aparasitemic controls.*** Plasma levels of MIF, IL-12 and TGF-β1 were determined by ELISA. MIF concentrations were log-transformed for normality. **(A)** Correlation of plasma MIF with IL-12 for children with acute malaria (Mal, n=21) and aparasitemic controls (AC, n=24). Pearson correlation: $r$=0.374, $P$<0.05 for Mal; $r$=0.214, $P$=0.2 for AC; $r$=0.216, $P$<0.05 for combined sample). **(B)** Correlation of plasma MIF with TGF-β1 for Mal (n=19) and AC (n=22). Pearson correlation: $r$=0.193, $P$=0.214 for Mal; $r$=0.303, $P$=0.08 for AC; $r$=0.271, $P$<0.01 for combined sample).

## 3.2 HYPOTHESIS 2, PRESENTATION OF MANUSCRIPT ENTITLED: *HIGHER PRODUCTION OF PERIPHERAL BLOOD MACROPHAGE MIGRATION INHIBITORY FACTOR IN HEALTHY CHILDREN WITH A HISTORY OF MILD MALARIA RELATIVE TO CHILDREN WITH A HISTORY OF SEVERE MALARIA.*

*Hypothesis 2: Elevated basal MIF production is protective against the development of severe malaria during subsequent P. falciparum infections.*

This hypothesis was addressed by examining plasma MIF levels and PBMC MIF transcripts in healthy aparasitemic Gabonese children enrolled in a longitudinal prospective study. The relationship between MIF production and severe malaria susceptibility was investigated by comparing children with different malaria disease histories: prior episodes of mild malaria and prior episodes of severe malaria. The results of these investigations are presented in the following manuscript published in the *American Journal of Tropical Medicine and Hygiene* (Am J Trop Med Hyg 2007, *76(6)*: 1033-1036; reproduced with permission form Am J Trop Med Hyg).

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Running title: Elevated MIF in healthy children with prior mild malaria

Keywords: Macrophage migration inhibitory factor (MIF), *Plasmodium falciparum*, severe malaria, immunity

### 3.2.1 Footnote page

Part of this work was presented at the 53rd Annual Meeting of the American Society of Tropical Medicine and Hygiene (ASTMH, Abstract # 775) held in Miami Beach, Florida USA (7th-11th November 2004). The study was approved by the Ethics Committee of the International Foundation of the Albert Schweitzer Hospital in Lambarènè, Gabon, Duke University Medical Center Institutional Review Board, and the University of Pittsburgh Institutional Review Board, and informed consent was obtained from all participants or the parents of participating children.

There is no conflict of interest for any of the authors of the manuscript due to either commercial or other affiliations.

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### 3.2.2 Abstract

*Plasmodium falciparum* malaria is one of the leading causes of childhood morbidity and mortality in sub-Saharan Africa. The host immune response to *P. falciparum* is a critical determinant of malarial pathogenesis and disease outcomes. Macrophage migration inhibitory factor (MIF) is a central regulator of innate immune responses to bacterial and parasitic infections. Our recent investigations demonstrated that peripheral blood MIF production was suppressed in children with severe malaria. Since examination of MIF production in children with active disease does not account for the inherent ability of the host to generate MIF, basal circulating MIF and peripheral blood mononuclear cell (PBMC) MIF transcript levels were determined in healthy children with a history of either mild or severe malaria. Children with prior mild malaria had higher plasma MIF levels and PBMC MIF transcripts than children with an identical number of previous episodes of severe malaria. Results presented here suggest that elevated basal MIF production may be important in generating immune responses that protect against the development of severe malaria.

### 3.2.3 Introduction, study participants, methods, results, and discussion.

Greater than one million people in sub-Saharan Africa die each year from *Plasmodium falciparum* malaria, with the majority of the deaths occurring in children less than 5 years of age (Guilbert 2003). The clinical manifestations of pediatric falciparum malaria vary from asymptomatic infection to severe life-threatening complications, such as hyperparasitemia, hypoglycemia, cerebral malaria, severe anemia, respiratory distress, and hyperlactatemia (WHO 2000; Taylor *et al.* 2006). A number of important factors appear to influence disease severity including host genetic variation, age of first exposure, and rate of exposure (endemicity) (Snow *et al.* 1997; Kwiatkowski 2005). In addition, it is well established that the host immune response to *P. falciparum* is an important determinant of the development and outcomes of childhood malaria (Clark and Cowden 2003; Clark *et al.* 2004).

As part of our ongoing studies examining the role of the host immune response in conditioning the outcomes of pediatric malaria, we have been investigating the role of macrophage migration inhibitory factor (MIF) in the pathogenesis of malaria.(Awandare *et al.* 2006b; Awandare *et al.* 2006c; Awandare *et al.* 2007b) Many studies have identified MIF as a central regulator of innate and adaptive immune responses that could mediate both protection and enhanced pathogenesis of bacterial and parasitic infections (Bernhagen *et al.* 1993; Bacher *et al.* 1996; Calandra *et al.* 1998; Juttner *et al.* 1998; Calandra *et al.* 2000; Koebernick *et al.* 2002; Calandra and Roger 2003; Reyes *et al.* 2006). Although previous studies in placental malaria and in murine models of malaria have suggested a pathogenic role for MIF in malaria (Martiney *et al.* 2000; Chaisavaneeyakorn *et al.* 2002; Chaiyaroj *et al.* 2004; McDevitt *et al.* 2006), our

recent studies demonstrate that increased circulating MIF production is associated with protection from severe childhood malaria (Awandare *et al.* 2006a; Awandare *et al.* 2007b).

To further clarify the role of MIF in malarial immunity, we examined MIF protein levels in circulation and MIF mRNA levels in peripheral blood mononuclear cells (PBMCs) from a group of healthy children (ages 2-8 years; mean age=6.3 years) enrolled in a longitudinal prospective study previously conducted at the Albert Schweitzer Hospital in Lambarènè, Gabon, a hyperendemic *P. falciparum* transmission area. In this region, hyperparasitemia and severe anemia are the predominant complications of severe malaria (Kun *et al.* 1998; Kun *et al.* 1998; Perkins *et al.* 1999). Study participants selected for investigation were in the convalescent phase of malaria and were free of malaria parasites and any other detectable diseases for four or more months based on bi-monthly malaria parasitemia determinations and clinical evaluations. A more detailed description of this study cohort is provided in our previous publication (Perkins *et al.* 1999). Based on their previous malaria disease history, children were divided into two groups: prior mild malaria (PMM, n=15; 8 males and 7 females; mean age of 6 years 3 months) or prior severe malaria (PSM, n=10; 6 males and 4 females; mean age of 6 years 4 months). Definitions of malaria disease severity during the acute illness were based on World Health Organization criteria (WHO 2000), with severe malaria defined as >200,000 parasites/μL and/or hemoglobin (Hb) ≤5.0g/dL and mild malaria defined as <100,000 parasites/μL, Hb >5.0g/dL, and without any signs or symptoms of severe malaria. None of the study participants had a prior episode of cerebral malaria. Children in the PMM and PSM groups were matched so that the mean number of previous malaria episodes was identical in the two groups. Moreover, children selected for the PMM group had no prior history of severe malaria, while those in the PSM group had no prior history of mild malaria. Participation in the study was completely voluntary and

written informed consent was obtained from the parents/guardians of the pediatric participants. The study was approved by the ethics committee of the International Foundation of the Albert Schweitzer Hospital, Duke University Medical Center Investigational Review Board, and the University of Pittsburgh Investigational Review Board. Venous blood (<5 mL) was collected into EDTA-containing vials, plasma was separated, and PBMC isolated according to our previous methods (Weinberg *et al.* 1981). At the time of sampling, a physical and clinical evaluation was performed to verify that the children were healthy and a thick blood smear was prepared to confirm that the study participants were free of malaria parasites. Plasma MIF concentrations were measured using commercially available ELISA (R&D systems, Minneapolis, MN). MIF transcripts were determined in *ex vivo* PBMC by Taqman® real time RT-PCR (Applied Biosystems, Foster City, CA) and normalized to β-actin according to our previous methods (Awandare *et al.* 2006b).

Healthy children were selected for investigation since differences in MIF production during an active infection may reflect the overall response to the pathogen, while baseline measurements in disease-free children in the convalescent phase of disease likely reflects either inherent genetic differences and/or adaptation to prior malaria episodes. As shown in Figure 7A, children with PMM [median (interquartile range), 1704 (1161-2497)] had significantly higher circulating MIF levels than children with PSM [1007 (887-1230), ($P$<0.005)]. In addition, MIF transcripts measured in *ex vivo* PBMC demonstrated that children with PMM had 2.4-fold higher MIF mRNA levels than children with PSM ($P$=0.2, Figure 7B).

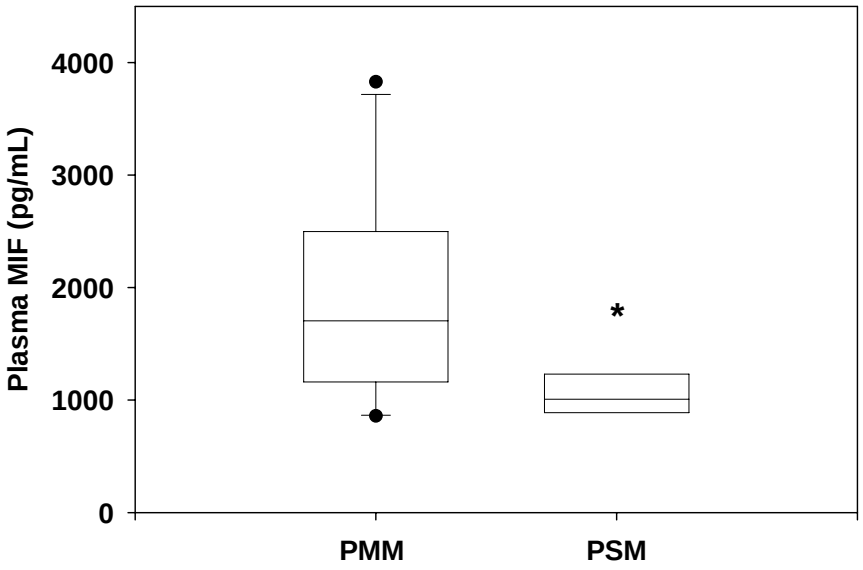
Unlike most cytokines, MIF is constitutively produced in significant quantities, and is further augmented by inflammatory stimuli (Bernhagen *et al.* 1998). Therefore, baseline MIF production may be important for determining the nature and magnitude of the host immune

response to an invading pathogen such as *Plasmodium*. Since samples examined in this study were taken from healthy children that had fully resolved their malarial infection, the observed levels of circulating MIF and PBMC MIF transcripts represent basal MIF production. Results presented here show that children with prior episodes of mild malaria produce significantly higher baseline levels of MIF than children that previously experienced severe disease. Given the pivotal role of MIF in mediating innate immunity and regulation of pro-inflammatory cytokine production (Juttner *et al.* 1998; Koebernick *et al.* 2002; Calandra and Roger 2003; Reyes *et al.* 2006), we propose that elevated baseline MIF levels may protect against the development of severe malaria by promoting a rapid and potent innate immune response that could result in more efficient control over the initial phases of parasitemia. For example, adequate MIF production is required for induction of interleukin-12, tumor necrosis factor-α and nitric oxide (NO) (Juttner *et al.* 1998; Koebernick *et al.* 2002; Reyes *et al.* 2006), all of which are important mediators of the innate immune response to malaria (Crutcher *et al.* 1995; Luty *et al.* 2000; Perkins *et al.* 2000; Chang and Stevenson 2004). In addition, since MIF is important in adaptive immunity through promotion of T and B cell activation and proliferation, and antibody production (Bacher *et al.* 1996) adequate MIF concentrations may be required for an efficient antigen-specific immune response to malaria. Conversely, elevated MIF in children with PMM may be related to the phenomenon of malarial tolerance (Clark and Cowden 2003; Clark *et al.* 2004) in which elevated MIF levels may provide negative-feedback mechanisms that help control over-expression of pro-inflammatory cytokines that could cause enhanced pathology. Our previous studies in the same cohort of healthy, malaria-exposed children illustrated that NO production was significantly higher in the PMM group (Perkins *et al.* 1999). This previous finding, along with results presented here are consistent with our hypothesis that elevated levels of regulatory inflammatory

mediators in children with prior malaria exposure may condition tolerance to malaria and reduce susceptibility to severe disease (Clark and Cowden 2003; Clark *et al.* 2004). Alternatively, differences in host genetics may explain our current findings. Two polymorphisms in the MIF promoter (MIF -173 G/C and MIF -794 $CAAT_{5\text{-}8}$) have been associated with functional changes in MIF production in a number of inflammatory diseases (Donn *et al.* 2001; Baugh *et al.* 2002; Donn *et al.* 2002; Donn *et al.* 2004). In addition, we recently demonstrated a significant association between circulating MIF levels and MIF -173 G/C variability in Kenyan children (Awandare *et al.* 2006c). Although polymorphic variability was not determined here, we are currently investigating the impact of MIF promoter polymorphisms on baseline and malaria-induced MIF production in pediatric populations.

We thank the following staff members of Albert Schweitzer Hospital in Lambaréné Gabon for technical assistance to the study: Dr. Anita van den Biggerlaar, Judith Jans, Dr. Hanna Knoop, Dr. Doris Luckner, Barbara Moritz, Anselme Ndzengue, Marcel Nkeyi, Dr. Daniela Schmid, and Dr. Milena Sovric. The study was conducted at the University of Pittsburgh, and supported in part by the National Institutes of Health Grant (AI-51305-01 DJP) and (AI-41764 JBW), the VA Research Service (JBW) and Fogarty International Training Grant (5D43-TW00588-4 DJP).

**A:**




**B:**

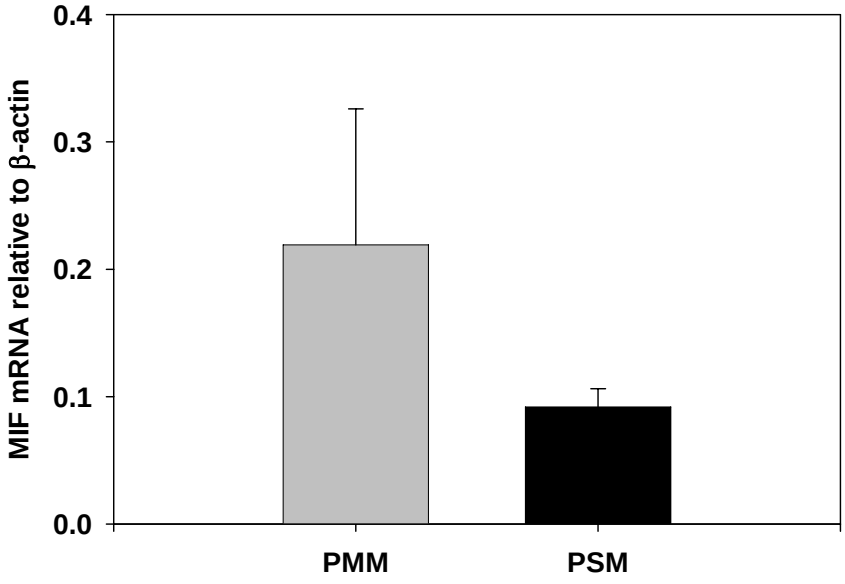



**Figure 7:** ***Circulating MIF and PBMC MIF mRNA in healthy children with prior mild malaria or prior severe malaria.***

***Figure 7: Circulating MIF and PBMC MIF mRNA in healthy children with prior mild malaria or prior severe malaria.*** Children were stratified into prior mild malaria (PMM, n=15) and prior severe malaria (PSM, n=10) based on their history of malaria disease severity. **(A)** Plasma levels of MIF are presented as box plots where the box represents the interquartile range, the line through the box is the median, whiskers indicate the $10^{th}$ and $90^{th}$ percentiles and individual symbols are outliers. *Differences in MIF levels in PMM compared to PSM were statistically significant ($P<0.005$, Mann-Whitney U-test). **(B)** Bars represent mean $\pm$ SEM of MIF mRNA relative to β-actin mRNA for the two groups. MIF mRNA levels in PSM (n=12) compared to PMM (n=8) were not statistically significant ($P=0.2$, Student's t-test). There was insufficient RNA obtained from PBMC of 5 children and as such, their samples failed to amplify during the RT-PCR.

## 3.3 HYPOTHESIS 3 AND 4, PRESENTATION OF MANUSCRIPT ENTITLED: *ROLE OF MONOCYTE-ACQUIRED HEMOZOIN IN SUPPRESSION OF MACROPHAGE MIGRATION INHIBITORY FACTOR IN CHILDREN WITH SEVERE MALARIAL ANEMIA.*

*Hypothesis 3: Peripheral blood MIF production is associated with anemia severity in children with acute P. falciparum malaria*

*Hypothesis 4: Phagocyte acquisition of malarial pigment (hemozoin) leads to suppression of MIF production during acute malaria*

The studies conducted in the Gabonese cohort were limited by the small sample size, which did not allow categorization of children according to disease severity. Therefore, to address *hypotheses 3*, peripheral blood MIF production was examined in a large cohort of Kenyan children presenting at a rural hospital with varying severities of malarial anemia and parasitemia. This study was conducted as part of a larger ongoing study examining the molecular, immunological and genetic basis of SMA at the Siaya district hospital in western Kenya, where *P. falciparum* transmission is holoendemic, and SMA and HDP are the primary clinical manifestations of severe malaria (McElroy *et al.* 2000; Ong'echa *et al.* 2006). Additional studies addressing *hypothesis 4* investigated the relationship between levels of pigment-containing monocytes and neutrophils, MIF production and SMA pathogenesis. Data resulting from these studies are presented in the following manuscript published in the journal *Infection and Immunity*

(Infect Immun 2007, 75: 201-210; reproduced with permission from the American Society for Microbiology).

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**Running title:** Hemozoin-induced MIF suppression in SMA

**Word counts:**

Abstract: 250

Text: 4531

### 3.3.1 Footnote page

A portion of this work was presented at the fourth Multilateral Initiative on Malaria (MIM) Pan-African malaria conference held in Yaoundé, Cameroon ($13^{th}$-$18^{th}$ November 2005). The study was approved by the Ethics Committees of the Kenyan Medical Research Institute (KEMRI), Kenyan Ministry of Health and the University of Pittsburgh Institutional Review Board, and informed consent was obtained from all participants or the parents/legal guardians of all participating children.

There is no conflict of interest for any of the authors of the manuscript due to either commercial or other affiliations.

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### 3.3.2 Abstract

Severe malarial anemia (SMA), caused by *Plasmodium falciparum* infections, is one of the leading causes of childhood mortality in sub-Saharan Africa. Although the molecular determinants of SMA are largely undefined, dysregulation in host-derived inflammatory mediators influences disease severity. Macrophage migration inhibitory factor (MIF) is an important regulator of innate inflammatory responses that has recently been shown to suppress erythropoiesis and promote pathogenesis of SMA in murine models. To examine the role of MIF in the development of childhood SMA, peripheral blood MIF production was examined in Kenyan children (aged <3 years, n=357) with *P. falciparum* malarial anemia. All children in the study were free from bacteremia and HIV-1. Since deposition of malarial pigment (hemozoin) contributes to suppression of erythropoiesis, the relationship between MIF concentrations and monocytic acquisition of Hz was also examined *in vivo* and *in vitro*. Circulating MIF concentrations declined with increasing severity of anemia and significantly correlated with peripheral blood leukocyte MIF transcripts. MIF concentrations in peripheral blood were not significantly associated with the reticulocyte production. Multivariate regression analyses, controlling for age, gender and parasitemia, further revealed that elevated levels of pigment-containing monocytes (PCM) was associated with SMA and decreased MIF production. In addition, PCM levels were a better predictor of hemoglobin and MIF concentrations than parasite density. Additional experiments in malaria-naïve individuals demonstrated that hemozoin caused both increased and decreased MIF production in cultured peripheral blood mononuclear cells (PBMC) in a donor-specific manner, independent of apoptosis. However, PBMC MIF production in children with acute malaria progressively declined with increasing anemia severity.

Results presented here demonstrate that acquisition of hemozoin by monocytes is associated with suppression of peripheral blood MIF production and enhanced severity of anemia in childhood malaria.

**Key words:** *Plasmodium falciparum*, malaria, anemia, MIF, malarial pigment (hemozoin)

### 3.3.3 Introduction

Annually, there are 350-500 million clinical cases of malaria that result in over one million deaths (WHO 2005). The majority of malaria-related mortality are due to infections with *Plasmodium falciparum* and occur in immune-naïve infants and young children in sub-Saharan Africa, accounting for 18% of all deaths before five years of age (WHO 2005). Severe malarial anemia (SMA) accounts for the greatest proportion of malaria related morbidity and mortality worldwide (Breman *et al.* 2001). In holoendemic *P. falciparum* transmission areas, SMA is the commonest clinical presentation of severe malaria in children less than three years of age, with cerebral malaria (CM) occurring only in rare cases (McElroy *et al.* 2000; Ong'echa *et al.* 2006).

Markedly reduced hemoglobin (Hb) concentrations in children with SMA result from overlapping, but distinct processes including direct and indirect destruction of parasitized red blood cells (pRBCs), increased clearance of uninfected erythrocytes, and suppression of erythropoiesis (Weatherall and Abdalla 1982; Abdalla 1990; Wickramasinghe and Abdalla 2000). Our recent studies in western Kenya, as well as those of others, illustrate that children with SMA (Hb<6.0g/dL) have lower peripheral parasite densities than parasitemic children without anemia (Hb ≥11.0 g/dL) (McElroy *et al.* 2000; Ong'echa *et al.* 2006), suggesting that acute hemolysis of RBCs is not likely responsible for the low Hb levels observed in children with SMA in this holoendemic region. It is well-documented that children with SMA have a number of bone marrow abnormalities including dyserythropoiesis, ineffective erythropoiesis, and reduced proliferation of erythroid colonies (Srichaikul *et al.* 1969; Weatherall and Abdalla 1982; Abdalla 1990; Wickramasinghe and Abdalla 2000). These findings, along with studies showing that the reticulocyte response is reduced relative to the degree of anemia in children

with malarial anemia, even in the presence of elevated levels of erythropoietin (Burchard *et al.* 1995; Kurtzhals *et al.* 1997; Nussenblatt *et al.* 2001), suggest that suppression of erythropoiesis may play a central role in the development of SMA.

Although the molecular mechanisms that regulate suppression of erythropoiesis are only partially defined, over-production of pro-inflammatory mediators is thought to play a prominent role in conditioning the severity of childhood malarial anemia (Clark *et al.* 1981; Kurtzhals *et al.* 1998; Othoro *et al.* 1999; Luty *et al.* 2000; Perkins *et al.* 2000; McDevitt *et al.* 2004). Recent investigations by our laboratory (Keller *et al.* 2004b; Ochiel *et al.* 2005; Awandare *et al.* 2006b; Were 2006) and others (Martiney *et al.* 2000; McDevitt *et al.* 2006) have focused on defining the role of pro-inflammatory mediators such as macrophage migration inhibitory factor (MIF) in the immunopathogenesis of malarial anemia, since MIF plays a pivotal role in regulating the innate immune response to invading pathogens (Calandra and Roger 2003). MIF is a pleiotropic cytokine released by several cell types including, monocytes/macrophages (Calandra *et al.* 1994), T cells (David 1966; Bacher *et al.* 1996), and cells of the anterior pituitary gland (Bernhagen *et al.* 1993). Although MIF has potent pro-inflammatory properties that protect against *Salmonella typhimurium* (Koebernick *et al.* 2002), *Leishmania major* (Juttner *et al.* 1998; Xu *et al.* 1998; Satoskar *et al.* 2001) and *Trypanosoma cruzi* (Reyes *et al.* 2006), elevated levels of circulating MIF are also associated with enhanced pathogenesis of bacterial sepsis (Bernhagen *et al.* 1993; Bozza *et al.* 1999; Calandra *et al.* 2000), suggesting that increased MIF production can elicit both protective and pathogenic responses in different infectious diseases.

Previous investigations have observed elevated levels of MIF protein in blood vessel walls of Malawian children with cerebral malaria (Clark *et al.* 2003; Clark and Cowden 2003), and intervillous blood mononuclear cells from women with placental malaria

(Chaisavaneeyakorn *et al.* 2002; Chaiyaroj *et al.* 2004). Studies in murine models of malaria show that elevated plasma MIF concentrations are associated with suppression of erythropoiesis and enhanced severity of anemia (Martiney *et al.* 2000; McDevitt *et al.* 2006). However, our recent investigations, which were the first to report circulating MIF levels in children with malaria, demonstrated that peripheral blood MIF concentrations and peripheral blood mononuclear cell (PBMC) MIF mRNA expression were reduced in Gabonese children with mild-to-moderate forms of malarial anemia and hyperparasitemia (Awandare *et al.* 2006b). In contrast, subsequent studies have shown that plasma MIF levels are elevated in Zambian children with malarial anemia (McDevitt *et al.* 2006).

Although the host-parasite interactions that mediate MIF production are largely undefined, studies from our laboratory (Perkins *et al.* 2003; Keller *et al.* 2004a; Keller *et al.* 2004b), and others (Pichyangkul *et al.* 1994; Sherry *et al.* 1995; Arese and Schwarzer 1997; McDevitt *et al.* 2006) show that ingestion of malarial pigment, hemozoin (Hz) by phagocytic cells causes dysregulation in cytokine production. Hz is a coordinated aggregation polymer of heme generated by *Plasmodia* during digestion of host Hb (Goldie *et al.* 1990). During a malaria infection, Hz is acquired by leukocytes through direct phagocytosis of pRBCs and free Hz released upon pRBCs rupture (Schwarzer *et al.* 1992). Recent studies have demonstrated that Hz contributes to the pathogenesis of SMA by suppressing erythropoiesis both directly and in synergy with pro-inflammatory cytokines such as TNF-α(Casals-Pascual *et al.* 2006). In addition, acquisition of Hz by murine macrophages and human monocytes is associated with enhanced MIF production (Martiney *et al.* 2000; McDevitt *et al.* 2006).

To investigate the role of MIF in the immunopathogenesis of malarial anemia, peripheral blood MIF concentrations and leukocyte MIF transcript levels were measured in infants and

young children residing in a holoendemic *P. falciparum* transmission area where severe anemia is the primary clinical manifestation of severe malaria. In addition, to examine host-parasite interactions that may be important for regulating MIF production, the relationship between circulating MIF levels and monocytic acquisition of Hz was determined. The direct effect of Hz on MIF production was also investigated in cultured PBMC from malaria-naïve individuals. *In vivo* and *in vitro* results presented here demonstrate that MIF is suppressed in children with SMA and that monocyte-acquired *pf*Hz plays an important role in promoting SMA and decreasing MIF production.

### 3.3.4 Study participants, materials, and methods

#### 3.3.4.1 *Study site.*

Children (n=357, age 3-31 mos) were recruited at the pediatric ward of the Siaya District Hospital (SDH), Nyanza Province, western Kenya. In this region, malaria is holoendemic with entomological inoculation rates of up to 300 per annum (Beier *et al.* 1994). Approximately 1/3 of all pediatric admissions to SDH are due to SMA, which accounts for ~50% of all pediatric deaths at hospital (Lackritz *et al.* 1992). A detailed description of the study site and cohort is provided in our recent report (McElroy *et al.* 2000; Ong'echa *et al.* 2006).

#### 3.3.4.2 *Study participants.*

A questionnaire and medical informatics system were used to recruit children at their first hospital contact for the treatment of malaria. After obtaining informed written consent from the parents/guardians of children presenting at SDH with the signs and symptoms of malaria, heel/finger-prick blood (<100 μL) was used to determine parasitemia and Hb concentrations. Children with *P. falciparum* parasitemia (any density) were categorized according to the following criteria: uncomplicated malaria (UM, Hb≥11.0 g/dL, n=26); mild malarial anemia (MlMA, 8.0≤Hb<11.0 g/dL, n=75), moderate malarial anemia (MdMA, 6.0≤Hb<8.0 g/dL, n=98); and SMA (Hb<6.0 g/dL, n=119). Case definitions of anemia were based on previous studies in western Kenya examining >10,000 longitudinal Hb measurements in an age- and geographically-matched population (McElroy *et al.* 2000). Healthy, aparasitemic children (AC, Hb≥11.0 g/dL, n=39) presenting at SDH for routine immunizations were recruited as controls. In addition, only those children that were afebrile for ≥2 weeks were included in the AC group.

None of the children in the current study had any signs/symptoms of CM. Since HIV-1 and bacteremia are common co-pathogens that influence anemia status in children with malaria (Akpede and Sykes 1993; Berkley *et al.* 1999; Graham *et al.* 2000; Otieno *et al.* 2006), and could influence MIF production, all study participants were screened for HIV-1 and/or bacteremia and those children found to have co-pathogens were excluded from all analyses. Pre- and post-test HIV counseling was provided to parents/guardians of all children enrolled. The study was approved by the Ethics Committees of the Kenya Ministry of Health and the University of Pittsburgh Institutional Review Board.

#### 3.3.4.3 *Sample collection.*

Prior to administration of antimalarials and/or any other treatment interventions, venous blood (<3 mL: a volume determined to be safe based on size, weight, and anemia status) was collected, and plasma isolated according to our previous methods (McElroy *et al.* 2000; Ong'echa *et al.* 2006). Leukocytes were obtained from the buffy coat by lysing RBCs followed by storage in RNAlater® (Ambion) at -20$^{o}$C until use.

#### 3.3.4.4 *Laboratory evaluation.*

Giemsa-stained blood smears were used to determine parasitemia and pigment-containing monocytes and neutrophils. The number of RBCs infected with asexual *P. falciparum* parasites were determined per 300 leukocytes, and peripheral parasite density/μL of blood was calculated using the WBC count/μL obtained on a Coulter® A$^{c}$T diff2™ (Beckman Coulter Corp.). A total of 30 monocytes and 100 neutrophils were examined per thin smear, and the number of pigment-containing monocytes (PCM) or pigment-containing neutrophils (PCN) was expressed as a percentage of the total number of monocytes and neutrophils, respectively (Nguyen *et al.* 1995;

Lyke *et al.* 2003). The number of PCM/μL of blood was calculated by multiplying the percent of PCM by the absolute number of monocytes/μL obtained from the Coulter® A$^c$T diff2™. Reticulocyte counts (percent) were determined using new methylene-blue-stained slides, and the absolute reticulocyte number (ARN) was calculated by multiplying the reticulocyte percent/100 by total RBC counts. HIV-1 status was determined per our previously described HIV-1 serology and PCR methods (Otieno *et al.* 2006), while bacteremia was determined using the Wampole™ ISOLATOR™ 1.5 mL microbial system (Inverness Medical).

**3.3.4.5** ***Determination of plasma MIF and leukocyte MIF transcript levels.***

To avoid the possible influence of MIF released from lysis of erythrocytes (Mizue *et al.* 2000) during blood clotting, circulating MIF levels were determined in plasma rather than serum samples. In addition, visibly hemolyzed samples were excluded from measurements. Plasma and culture supernatant MIF concentrations were determined by ELISA with a matched anti-MIF antibody pair (R&D systems). All samples were assayed at 1:5 and 1:10 dilutions in duplicate, and according to manufacturer's recommendations. The limit of detection was >31.25 pg/mL. For determination of MIF transcript levels, total RNA was extracted from leukocyte pellets using the guanidinium isothiocyanate (GITC) method as described previously (Chomczynski and Sacchi 1987). Fluorogenic primer/probe sets specific for MIF and the housekeeping gene, β-actin (assay IDs Hs00236988_g1 and 4326315E respectively, Applied Biosystems) were used for real time RT-PCR on an ABI Prism® 7700 sequence detection system (Applied Biosystems). MIF mRNA levels were normalized by expressing transcripts as fold-change relative to β-actin mRNA ($2^{-\Delta CT}$, where $\Delta C_T$=critical threshold cycle of MIF – critical threshold cycle for β-actin), as described previously (Awandare *et al.* 2006b).

#### 3.3.4.6 *PBMC cultures.*

Venous blood was obtained from healthy, malaria-naïve US donors (n=15) and children with acute malaria (n=94). PBMC were isolated using Ficoll-Hypaque according to previous methods (Weinberg *et al.* 1981). To ensure complete removal of RBCs, PBMC were treated with RBC lysis buffer (BioWhittaker) for five minutes and then washed prior to culture. *P. falciparum*-derived Hz (*pf*Hz) was isolated from laboratory-cultivated *P. falciparum* (PfD6) and synthetic Hz (sHz) was prepared from hemin chloride (Sigma) as described previously (Keller *et al.* 2004b). Endotoxin levels in all *pf*Hz and sHz preparations were determined to be <0.125 U/mL (i.e., <0.025 ng/mL; *Limulus amebocyte* lysate test, BioWhittaker). PBMC were plated at $1\times10^6$ cells/mL in Dulbecco's modified Eagles medium (DMEM) containing HEPES buffer (25mM), penicillin (100 U/mL)/streptomycin (100 μg/mL) and 10% heat inactivated human serum from a non-malarious region. Experiments conducted in children with acute malaria were cultured in media alone, while PBMC from US donors were stimulated with media alone (unstimulated control), physiological concentrations of *pf*Hz (10 μg/mL) (Keller *et al.* 2004b), sHz (10 μg/mL), or lipopolysaccharide (LPS, 100 ng/mL; Alexis Corp.) and interferon (IFN)-γ (200 U/mL; Biosource).

#### 3.3.4.7 *Cell viability and apoptosis assays.*

Viability of cultured PBMC was determined using a methylthiazoletetrazolium (MTT)-based assay according to manufacturer's recommendations (Sigma). Cellular apoptosis was assessed by quantifying the concentrations of nucleosomes in cell lysates (early-stage apoptosis) and culture supernatants (late-stage apoptosis) using a cell death detection ELISA (Roche

Diagnostics) according to manufacturer's recommendations, with nucleosome concentrations in treated cells expressed as a percentage relative to untreated cells (control).

#### 3.3.4.8 *Statistical analyses.*

Kruskal-Wallis tests were used to compare variables across three or more groups, and where significant differences were observed, Mann-Whitney U tests were conducted for pairwise comparisons. Statistical associations between variables were examined using Pearson's correlation tests, and multivariate linear and logistic regression analyses, controlling for age and gender. Prior to performing Pearson's correlational analyses, the distributional characteristics of all variables were examined for departures from normality using the Kolmogorov-Smirnov test. Those variables with significant skewness were transformed toward normality. *P*-values less than 0.05 were considered statistically significant for all analyses.

### 3.3.5 Results

#### 3.3.5.1 *Clinical, parasitological, and hematological characteristics of study participants.*

To examine the role of MIF in the immunopathogenesis of malarial anemia, children (n=357) were stratified into clinical anemia categories. The clinical, parasitological, and hematological characteristics of the study participants upon admission are summarized in Table 3. Across-group comparisons showed significant differences in age ($P$=0.005) and axillary temperature ($P$<0.001). Children in the UM group were significantly older relative to all other groups ($P$<0.01 for all comparisons). Axillary temperature was elevated in all categories of children with acute malaria relative to the AC group ($P$<0.001 for all groups). Mean peripheral parasitemia ($P$=0.117), geometric mean parasitemia, and the proportions of children with HDP (≥10,000 parasites/μL; $P$=0.656) were not significantly different among children with acute malaria (Table 3). Since anemia severity was the basis for classification, Hb and RBC numbers decreased across the groups ($P$<0.001 for both comparisons). In contrast, the ARN increased with increasing severity of anemia ($P$<0.001). Taken together, these data illustrate that the severity of anemia in this holoendemic *P. falciparum* transmission region is not significantly associated with peripheral parasite density.

#### 3.3.5.2 *Circulating MIF levels progressively decline with increasing severity of malarial anemia.*

Our previous investigations showed that peripheral blood MIF concentrations were suppressed in a small cohort of children with acute malaria (Awandare *et al.* 2006b) . In contrast, additional studies (also limited by sample size) illustrated that plasma MIF concentrations were elevated in

children with acute malaria (McDevitt *et al.* 2006). To more fully investigate the role of MIF in the immunopathogenesis of childhood malaria, children were stratified according to malarial anemia status and circulating MIF levels were examined in the following groups: UM ($n$=23), MlMA ($n$=71), MdMA ($n$=94) and SMA ($n$=109); and healthy, aparasitemic controls (AC, n=39). As shown in Figure 8A, MIF concentrations progressively declined in parasitemic children with increasing severity of anemia. Children with SMA [median (interquartile range) 3422 (1566-4993) pg/mL)] had lower plasma MIF levels than the AC [4383 (2807-6376) pg/mL, $P$<0.05], UM [5225 (3615-9071) pg/mL, $P$<0.001], and MlMA [4611 (3270-6665) pg/mL, $P$<0.001] groups (Figure 8A). In addition, there was a significant positive correlation between circulating MIF levels and Hb concentrations ($r$=0.23, $P$<0.001) and a significant inverse correlation between plasma MIF levels and parasitemia ($r$=-0.16, $P$<0.05) among children with malaria (Figures 8B and C). However, there was a non-significant correlation between circulating MIF concentrations and the absolute reticulocyte number (r=0.10, $P$=0.10, Figure 8D). Multiple regression analysis, controlling for age and gender, further revealed that MIF was a significant positive predictor of Hb concentration (standardized partial regression coefficient, β-weight=0.153, $P$<0.005) and a negative predictor of parasitemia (β-weight=-0.096, $P$=0.067). These results demonstrate that SMA is characterized by reduced peripheral blood MIF concentrations and that pRBCs and/or a parasitic product(s) either directly or indirectly promote suppression of MIF concentrations.

**3.3.5.3** ***Peripheral blood leukocyte MIF transcripts as a source of circulating MIF.***

Although MIF is produced by a number of different cell types (Bernhagen *et al.* 1993; Calandra *et al.* 1994; Calandra and Roger 2003), our recent investigations in Gabonese children with malaria demonstrated that suppression of plasma MIF concentrations was associated with

decreased PBMC MIF transcripts (Awandare *et al.* 2006b). Based on the number of different experimental measures performed with limited blood sample volumes from children with malarial anemia, we opted to measure MIF transcripts in peripheral blood leukocytes (PBL) from a subset of children in which matched samples were available. These experiments revealed that MIF transcripts closely paralleled circulating MIF concentrations ($r$=0.68, $P$=0.01; Figure 9), suggesting that MIF gene expression in PBL may be an important source of circulating MIF levels.

#### 3.3.5.4 *MIF production from cultured PBMC progressively declines with increasing anemia severity.*

To further investigate MIF production in children with malarial anemia, PBMC MIF production was measured under baseline conditions in cultured cells isolated from children with varying degrees of malarial anemia and healthy controls: AC (n=24), UM (n=15), MlMA (n=37), MdMA (n=13), and SMA (n=5). MIF concentrations in culture supernatants taken after 48 hrs were significantly increased in children with UM [median (interquartile range) 2218 (1418-3989) pg/mL)] relative to the AC group [1034 (615-1902) pg/mL, $P$<0.005, Figure 10). However, MIF production progressively declined with increasing anemia severity, with the SMA group [511 (264-2312) pg/mL)] having lower culture supernatant MIF levels than the UM ($P$<0.05), MlMA [1609 (940-2439) pg/mL, $P$=0.08], and MdMA [842 (495-3737) pg/mL, $P$=0.43] groups (Figure 10). There was no significant difference in PBMC MIF production in any of the malarial anemia groups relative to AC (SMA, $P$=0.51; MdMA, $P$=0.87 and MlMA, $P$=0.06). Taken together, these *in vitro* experiments demonstrate that reduced MIF production from PBMC contributes to decreased circulating MIF levels in children with malarial anemia. Furthermore, increased MIF

production in children with a good clinical outcome, i.e., the UM group, suggests that an adequate MIF response may provide protection against severe malaria.

**3.3.5.5** ***In vivo acquisition of pfHz by monocytes is associated with enhanced SMA.***

Recent studies in individuals with malaria suggest that phagocytosis of *pf*Hz by monocytes and neutrophils is an important predictor of clinical severity (Nguyen *et al.* 1995; Amodu *et al.* 1998; Lyke *et al.* 2003; Casals-Pascual *et al.* 2006). Additional studies have demonstrated that sHz enhanced MIF production in mouse peritoneal macrophages and human mononuclear cells (McDevitt *et al.* 2006). To further examine the role of naturally acquired *pf*Hz in conditioning disease outcomes in children with malarial anemia, the relationship between intracellular malarial pigment and malaria disease severity was determined. Percentages of PCM ranged from 0-90% (mean=10%), while the percentages of PCN ranged from 0-9% (mean=0.3%), with PCM present in 47% (144/306) and PCN present in 12% (37/306) of the study participants, respectively. Based on the higher abundance of PCM relative to PCN, we examined the impact of PCM on clinical outcomes. The proportion of pigment-containing monocytes (%PCM) and PCM/μL progressively increased across the acute malaria groups ($p<0.001$ for both comparisons, see Table 3). In a multiple regression model controlling for age, gender, and parasitemia, PCM emerged as a strong predictor of Hb concentrations (β-weight=-0.434, $P<0.001$).

To further investigate the role of PCM in conditioning the outcomes of acute malaria, children were stratified into three groups according to the distribution of PCM: *no PCM* (0% PCM), *low PCM* (PCM≤10%), and *high PCM* (PCM>10%). These analyses revealed that age was not significantly different across the groups ($P=0.645$). Moreover, although parasitemia increased with elevated PCM across the three groups ($P=0.034$, Table 4), parasitemia and the

percentage of HDP did not significantly differ between the *high* and *low* PCM groups ($P$=0.181 and $P$=0.086, respectively, Table 4). Conversely, Hb concentrations decreased with increasing *pf*Hz deposition ($P$<0.001, across groups), with the *high PCM* group having the highest percentage of SMA cases (Table 4). Multiple logistic regression analyses controlling for age, gender, and parasitemia demonstrated that the risk of SMA was increased for the *low PCM* (OR=3.4; 95% CI, 1.8-6.6, $P$<0.0001) and *high PCM* (OR=7.5; 95% CI, 4.1-14.0, $P$<0.0001) groups relative to the *no PCM* group (Table 4). There was also an increased risk of SMA in the *high PCM* group compared to the *low PCM* group (OR=2.1; 95% CI, 1.1-4.3, $P$<0.05). Thus, consistent with the demonstrated role of Hz in suppression of erythropoiesis during malarial anemia (Casals-Pascual *et al.* 2006), these results show that deposition of *pf*Hz in circulating monocytes is associated with an increased prevalence of SMA.

**3.3.5.6** ***In vivo acquisition of pfHz by monocytes is associated with suppression of MIF.***

Since PCM was strongly correlated with SMA, and MIF concentrations declined with increasing severity of anemia, the relationship between PCM and MIF was examined. The *high PCM* group had lower MIF levels [median (interquartile range), 3115 (1525-4879) pg/mL] than the *no PCM* [4417 (3112-6266) pg/mL; $P$<0.005] and *low PCM* groups [4584 (1525-4879) pg/mL; $P$<0.05, Figure 11A]. Furthermore, circulating MIF concentrations were significantly inversely correlated with PCM levels ($r$=-0.23, $P$<0.001; Figure 11B). Multiple linear regression analyses controlling for age and gender further revealed that PCM (β-weight=-0.238, $P$<0.001) was a stronger predictor of MIF than parasitemia (β-weight=-0.087, $P$=0.024), suggesting that monocytic acquisition of *pf*Hz may contribute to suppression of MIF concentrations.

**3.3.5.7** ***pfHz and sHz decrease MIF production in cultured PBMC independent of apoptosis.***

Since the *in vivo* results suggested that PCM may be an important factor for suppressing MIF production, the direct effect of malarial pigment on MIF production was examined in PBMC from healthy, malaria-naïve U.S donors. Cultures were stimulated with concentrations of *pf*Hz or sHz comparable to those in children with severe malaria (i.e., 10 μg/mL) (Keller *et al.* 2004b), and LPS and IFN-γ (L/I) as a positive control. Treatment of PBMC with *pf*Hz or sHz resulted in a significant increase in MIF production in 3/15 donors ($P<0.05$ for all comparisons; Group 1, Figure 12A), while MIF production was significantly decreased in 11/15 donors ($P<0.05$ for all comparisons; Group 2, Figure 12A). In addition, there was no significant change in MIF production in *pf*Hz- or sHz-stimulated PBMC from one donor (data not shown). Stimulation of PBMC with L/I increased MIF production in 9/15 donors ($P<0.05$ for all comparisons, Figure 12A), while MIF production was not significantly altered in PBMC of 6/15 donors (data not shown).

Since *pf*Hz or sHz could alter MIF by influencing PBMC viability, cellular metabolism was investigated with the MTT assay. Relative to control conditions, stimulation with *pf*Hz or sHz had no significant effect on cellular viability at 48 hrs ($P=0.601$ and $P=0.522$, respectively), while L/I reduced viability by 28% ($P<0.05$; Figure 12B). Examination of cellular apoptosis by measuring nucleosome release (DNA fragmentation) revealed that both early- and late-stage apoptosis were unaffected by sHz treatment ($P=0.247$, and $P=0.928$, respectively). However, stimulation with L/I increased both early and late apoptosis ($P<0.001$ for both comparisons, Figure 12C) which may explain the lack of significantly enhanced MIF production in some of the donors. Taken together, these results show that malarial pigment decreases MIF production from PBMC, independent of altered cell viability or apoptosis.

### 3.3.6 Discussion

A number of studies in murine models of malaria suggest that there is a circulating host-derived inflammatory mediator(s) that inhibits the erythropoietic response (Silverman *et al.* 1987; Miller *et al.* 1989; Yap and Stevenson 1991), and recent evidence has identified MIF as that potential immune mediator that suppresses erythropoiesis during malaria (Martiney *et al.* 2000; McDevitt *et al.* 2006). However, the role of MIF in regulating anemia in childhood malaria remains unclear because of contradictory findings in small cohorts of children (Awandare *et al.* 2006b; McDevitt *et al.* 2006). While our previous study demonstrated suppression of circulating MIF and *ex vivo* PBMC MIF transcripts in Gabonese children with acute malaria versus healthy, aparasitemic controls (Awandare *et al.* 2006b), subsequent investigations illustrated that plasma MIF levels were elevated in Zambian children with malarial anemia relative to healthy controls (McDevitt *et al.* 2006).

To further investigate the role of MIF in the immunopathogenesis of malarial anemia, the relationship between MIF production and anemia was investigated in a large cohort of clinically well-characterized children that were stratified according to the severity of malarial anemia. These studies confirmed our previous finding in Gabon (Awandare *et al.* 2006b) and extended this observation by demonstrating that circulating MIF levels and MIF production from PBMC isolated from children with acute malaria progressively declined with increasing severity of malarial anemia. Results presented here also illustrated that there was a significant positive correlation between circulating MIF levels and Hb concentrations. Moreover, the large sample size in the current study allowed us to confirm the significant positive association between MIF and Hb by conducting multivariate regression analyses that controlled for important confounders

such as age. Our previous studies in this cohort of children demonstrated that the reticulocyte response was inappropriate for the level of anemia, and that children with malarial anemia had suppression of erythropoiesis (Were 2006). Correlation analyses examining the relationship between MIF and erythropoiesis revealed that MIF was not significantly associated with the absolute reticulocyte number, suggesting that circulating MIF may not be responsible for suppression of erythropoiesis.

The basis of different MIF production patterns in Zambian children (McDevitt *et al.* 2006) versus those in Gabon (Awandare *et al.* 2006b) and in Kenyan children presented here remains to be defined. However, our studies in western Kenya and those of others (Akpede and Sykes 1993; Berkley *et al.* 1999; Graham *et al.* 2000; Otieno *et al.* 2006) have shown that children presenting with malarial anemia often have co-infections with bacteremia and HIV-1 which could potentially alter MIF production. Although children with co-infections were excluded from our analyses, it is not certain if such co-infected children were excluded from other studies. In addition, since RBCs contain substantial quantities of MIF (Mizue *et al.* 2000), the use of plasma from hemolyzed blood could yield falsely elevated MIF concentrations. For analysis of circulating MIF in the current study, all plasma samples were examined by an individual who was blinded to the clinical categories of study participants, and those samples with evidence of hemolysis were excluded from measurement. Furthermore, it is well documented that malaria transmission intensity conditions the host immune response and age-specific pathophysiological manifestations of *P. falciparum* (Marsh and Snow 1997; Snow *et al.* 1997). The relatively older children examined in Zambia (mean age: 25.5 mos.), where *P. falciparum* endemicity is lower, therefore, may have different immunopathogenic mechanisms

responsible for the promotion of malarial anemia than the younger children examined here in western Kenya (see Table 1) residing in a holoendemic transmission area.

Recent investigations illustrated that MIF suppresses erythropoietin-dependent colony formation in cultured erythroid progenitor cells and Hb synthesis in murine and human cell lines (McDevitt *et al.* 2006). Additional studies showed that elevated MIF concentrations are present in bone marrow lysates of *P. chabaudi*-infected mice (Martiney *et al.* 2000). However, measurement of MIF concentrations in the bone marrow of children with SMA will be important for understanding the role of MIF in human malaria since contrasting roles of MIF in murine studies (Martiney *et al.* 2000; McDevitt *et al.* 2006) versus those in human is not unprecedented. For example, previous investigations revealed that MIF production in response to glucocorticoids differs in murine and human systems (Isidori *et al.* 2002; Alourfi *et al.* 2005). Furthermore, while anemia in murine models of malaria closely correlate with peripheral parasitemia and RBC hemolysis, data presented here illustrate that parasite density and anemia severity are unrelated in children with SMA. It is also important to note that MIF production during malaria appears to be both tissue- and compartment-specific (Chaisavaneeyakorn *et al.* 2002; Clark *et al.* 2003; Chaisavaneeyakorn *et al.* 2005). For example, previous studies in women with placental malaria showed increased MIF locally in intervillous blood in the presence of decreased peripheral blood MIF concentrations (Chaisavaneeyakorn *et al.* 2002). Therefore, our findings do not exclude the possibility that MIF may be increased in the local milieu within the bone marrows of the children in our cohort despite the reduced concentrations of MIF in the peripheral circulation.

Previous studies in malaria-endemic regions illustrate that phagocytosis of *pf*Hz by monocytes and neutrophils is a better index of disease severity than peripheral parasitemia (Metzger *et al.* 1995; Nguyen *et al.* 1995; Amodu *et al.* 1998; Lyke *et al.* 2003; Casals-Pascual *et*

*al.* 2006). This may be related to the fact that concomitant peripheral parasitemia does not account for parasite sequestration within microvascular networks and/or the duration of infection. In this study, there was a lower prevalence of PCM and PCN than previously observed by others (Metzger *et al.* 1995; Amodu *et al.* 1998; Lyke *et al.* 2003). This may be because children in the present study were younger and, therefore, likely more immune-naïve to *P. falciparum*. Importantly, the clearance kinetics of PCM and PCN differ considerably, with median PCM and PCN clearance times of approximately ten and four days, respectively (Day *et al.* 1996). Increased abundance of PCM relative to PCN in the current cohort suggests that malarial anemia results from prolonged (chronic) malaria infections. Although Hz has been shown to regulate MIF production *in vitro* (Awandare *et al.* 2006c; McDevitt *et al.* 2006), the relationship between MIF production and Hz levels *in vivo* has not previously been examined. Multivariate analyses controlling for age, gender, and parasitemia revealed a significant association between increased levels of PCM, SMA, and decreased circulating MIF levels, suggesting that acquisition of *pf*Hz by monocytes may promote SMA and suppress peripheral MIF production.

Based on the statistical relationship between elevated PCM and decreased MIF, malarial pigment was investigated as a parasitic source responsible for MIF suppression in children with SMA. A series of experiments in cultured PBMC from malaria-naïve donors identified two main types of responders: those who increased MIF production and those who decreased MIF production in response to stimulation by physiological doses (Keller *et al.* 2004b) of *pf*Hz or sHz. Cell viability experiments further demonstrated that reductions in MIF production were not mediated by loss in cell viability or increased apoptosis. Identical results observed with *pf*Hz and sHz demonstrate that the core ferriprotoporphyrin-IX structure, rather than adherent host

and/or parasitic proteins, lipids or nucleic acids is responsible for altering MIF production. Recent studies in cultured mononuclear cells from individuals with low (5-CATT/5-CATT) and high expression MIF-794 alleles (6-CATT/6-CATT and 6-CATT/7-CATT) show that MIF production in response to sHz stimulation was genotype-dependent (McDevitt *et al.* 2006). Although the genetic background of the donors examined in the present study were unknown, subsequent investigations in our laboratories have revealed that PBMC from individuals with the GG genotype at MIF -173 have increased MIF production, while GC individuals have decreased MIF production following stimulation with *pf*Hz (Awandare *et al.* 2006c). It is, therefore, not surprising that circulating MIF concentrations were generally inversely correlated with PCM levels in the study cohort, since the C allele is predominant in this cohort of children (Awandare *et al.* 2006c). The molecular mechanism(s) by which acquisition of malarial pigment decreases MIF production remains to be defined. However, previous studies demonstrated that ingestion of Hz causes impairment of several cellular functions including cytokine secretion, phagocytosis, and antigen-presentation (Schwarzer *et al.* 1992; Schwarzer *et al.* 1998). It remains to be determined if suppression of MIF in response to Hz occurs through a direct or indirect mechanism following phagocytosis of Hz. Taken together, results presented here, which represent the most comprehensive examination to date on the role of MIF in promoting childhood malarial anemia, suggest that MIF may not be responsible for enhanced anemia in pediatric populations with acute falciparum malaria, and that monocytic ingestion of Hz is responsible, either directly or indirectly, for suppression of MIF.

### 3.3.7 Acknowledgement

We are very grateful to all the parents, guardians and children from the Siaya District community and the US donors for their participation in this study. We would also like to thank the University of Pittsburgh/KEMRI staff and the Siaya District Hospital staff for their support during the study. We thank the Director of KEMRI for approving this manuscript for publication.

**Table 3:** ***Clinical, parasitological, and hematological characteristics of study participants.***

| Characteristic | AC | UM | MlMA | MdMA | SMA | *P*-value |
|---|---|---|---|---|---|---|
| Number, n | 39 | 26 | 75 | 98 | 119 | |
| Age, mos | 9.1 (1.2) | 14.0 (1.9) | 12.2 (0.8) | 11.5 (0.6) | 10.2 (0.6) | 0.005[a] |
| Axillary temperature, $^{o}$C | 36.8 (0.2) | 37.6 (0.2) | 37.6 (0.1) | 37.7 (0.1) | 37.6 (0.1) | <0.001[a] |
| Parasites/μL | 0 | 84118 (22734) | 44168 (6092) | 48697 (5925) | 74490 (10443) | 0.117[a] |
| Geomean parasites/μL | 0 | 29689 | 21020 | 18623 | 31458 | |
| HDP, n (%) | 0 | 20 (77) | 53 (71) | 71 (72) | 93 (78) | 0.656[b] |
| Hemoglobin, g/dL | 11.7 (0.1) | 11.8 (0.2) | 9.2 (0.1) | 6.9 (0.1) | 4.7 (0.1) | <0.001[a] |
| Hematocrit, % | 35.2 (0.3) | 34.5 (1.1) | 28.0 (0.4) | 21.7 (0.2) | 15.1 (0.3) | <0.001[a] |
| RBC, x $10^6$/μL | 4.91 (0.08) | 4.78 (0.13) | 4.15 (0.07) | 3.23 (0.06) | 2.18 (0.05) | <0.001[a] |
| ARN, x $10^3$/μL | 97.5 (18.7) | 105.7 (16.5) | 110.6 (8.7) | 147.3 (11.6) | 140.7 (10.4) | <0.001[a] |
| PCM, % | - | 0.8 (0.8) | 3.1 (1.0) | 7.6 (1.4) | 15.2 (1.6) | <0.001[a] |
| PCM/μL | - | 0.01 (0.01) | 0.04 (0.02) | 0.09 (0.02) | 0.23 (0.03) | <0.001[a] |

Data are presented as mean (SEM) except where otherwise indicated. Comparisons of parasitemia were among parasitemic groups only.

[a]Kruskal-Wallis test

[b]Chi-Square test

AC, aparasitemic controls (*P. falciparum* negative, Hb≥11.0 g/dL); UM, uncomplicated malaria (Hb≥11.0 g/dL); MlMA, mild malarial anemia (8.0≤Hb<11.0 g/dL); MdMA, moderate malarial anemia (6.0≤Hb<8.0 g/dL); SMA, severe malarial anemia (Hb<6.0 g/dL). HDP, high density parasitemia (≥10,000 parasites/μL); RBC, red blood cells; ARN, absolute reticulocyte number; PCM, pigment-containing monocytes.

**Table 4:** ***Relationship between monocyte acquisition of hemozoin and disease severity in children with malaria.***

| **Characteristic** | **Percent pigment-containing monocytes (PCM)** | | | |
|---|---|---|---|---|
| | ***No PCM (0%)*** | ***Low PCM (≤10%)*** | ***High PCM (>10%)*** | ***P-value*** |
| Number, *n (%)* | 162 (53) | 62 (20) | 82 (27) | |
| Age, *mos* | 11.3 (0.5) | 12.2 (0.9) | 11.1 (0.8) | 0.645[a] |
| Parasitemia, */μL* | 49371 (5088) | 60141 (9906) | 80447 (13266)[b] | 0.034[a] |
| HDP, *n (%)* | 113 (70) | 50 (79) | 67 (82) | 0.086[c] |
| Hemoglobin, *g/dL* | 7.8 (0.2) | 6.5 (0.2) | 5.7 (0.2)[d] | < 0.001[a] |
| SMA, *n (%)* | 34 (21) | 28 (46) | 53 (65)[d] | < 0.001[b] |
| Odds of SMA[e] | 1 | 3.4 (1.8-6.6) | 7.5 (4.1-14.0) | |

Data are presented as mean (SEM) unless otherwise indicated.

[a]Kruskal-Wallis test

[b]Significantly different from *no* PCM group, but not *low* PCM group

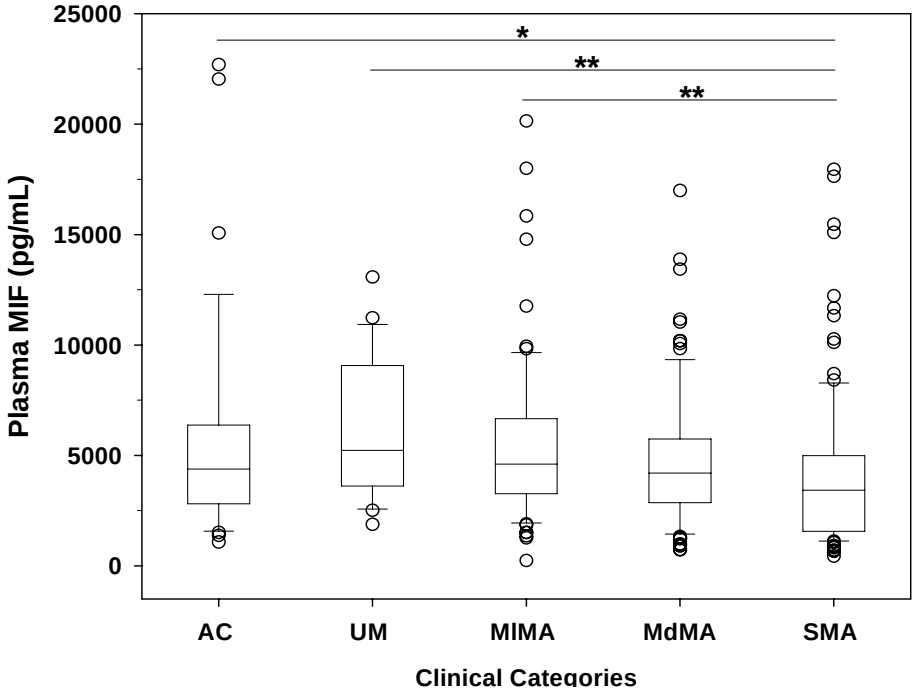
[c]Chi-Square test

[d]Significantly different from *No PCM* and *low PCM* groups.

[e]Multivariate logistic regression using *No PCM* group as reference and controlling for age, gender, and parasitemia; shown as odds ratio (95% confidence interval), $P<0.0001$ for both *low* and *high* PCM groups.

HDP, high density parasitemia (≥10,000 parasites/μL); SMA, severe malarial anemia (Hb<6.0 g/dL).

**A.**




**B.**

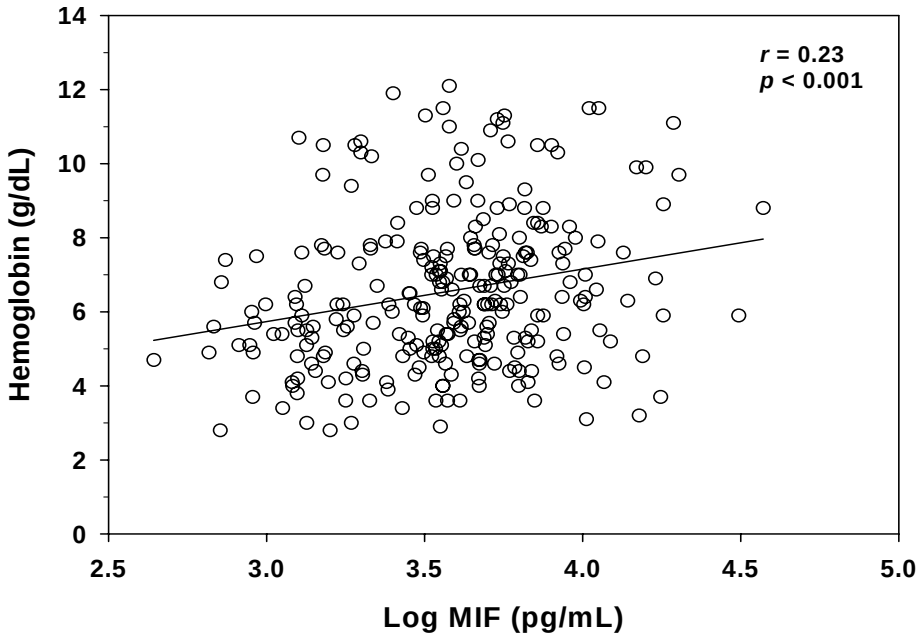
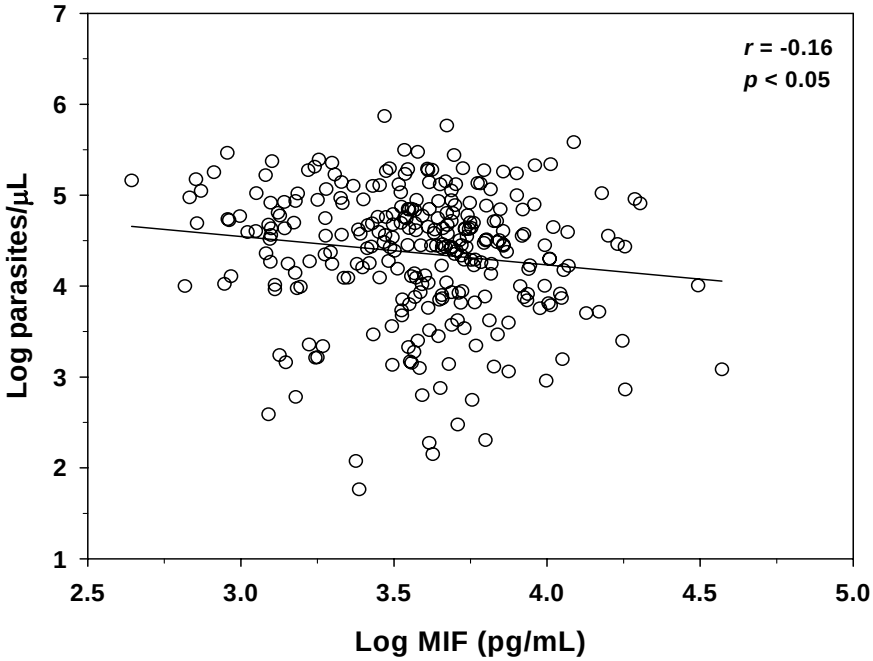



**Figure 8:** ***Relationship of plasma MIF with anemia, parasite density and reticulocyte number.***

C.




D.

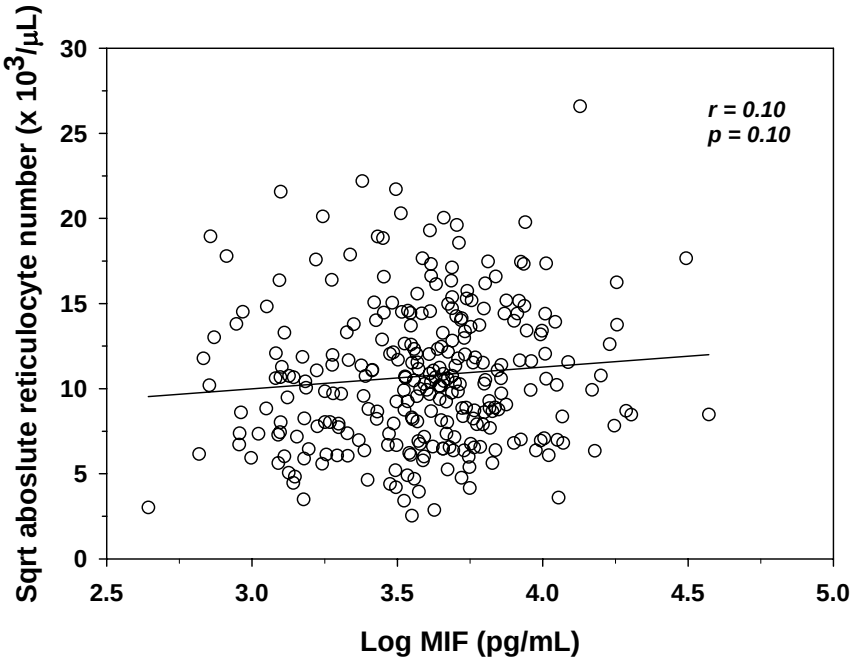



**Figure 8.** ***Relationship of plasma MIF with anemia, parasite density and reticulocyte number.***

**Figure 8.** ***Relationship of plasma MIF with anemia, parasite density and reticulocyte number.***

Plasma levels of MIF in children with malaria and controls were determined by ELISA. (**A**) Data are presented according to anemia categories: uncomplicated malaria (UM, Hb≥11.0 g/dL, n=23), mild malarial anemia (MlMA, 8.0≤Hb<11.0 g/dL, n=71), moderate MA (MdMA, 6.0≤Hb<8.0 g/dL, n=94) and severe MA (SMA, Hb<6.0 g/dL, n=109). Aparasitemic controls (AC, n=39) with Hb≥11.0 g/dL were used as a reference group. Boxes represent the interquartile range, the line through the box is the median, whiskers show $10^{th}$ and $90^{th}$ percentiles, and symbols are outliers. Median (interquartile range) levels of MIF were: AC, 4383 (2807-6376); UM, 5225 (3615-9071); MlMA, 4611 (3270-6665); MdMA, 4197 (2862-5743); and SMA, 3422 (1566-4993). **P*<0.05, ***P*<0.001; Mann-Whitney U tests conducted after analysis of variance (Kruskal-Wallis test) revealed significant differences across groups. Linear relationships of plasma MIF levels with hemoglobin concentrations (**B**), parasite density (**C**), and absolute reticulocyte number (ARN) (**D**), in children with malaria (n=298) are shown as scatter plots. MIF concentrations and parasitemia were log-transformed while ARN was square root (sqrt)-transformed for normality, and statistical association was determined by Pearson's correlation tests.

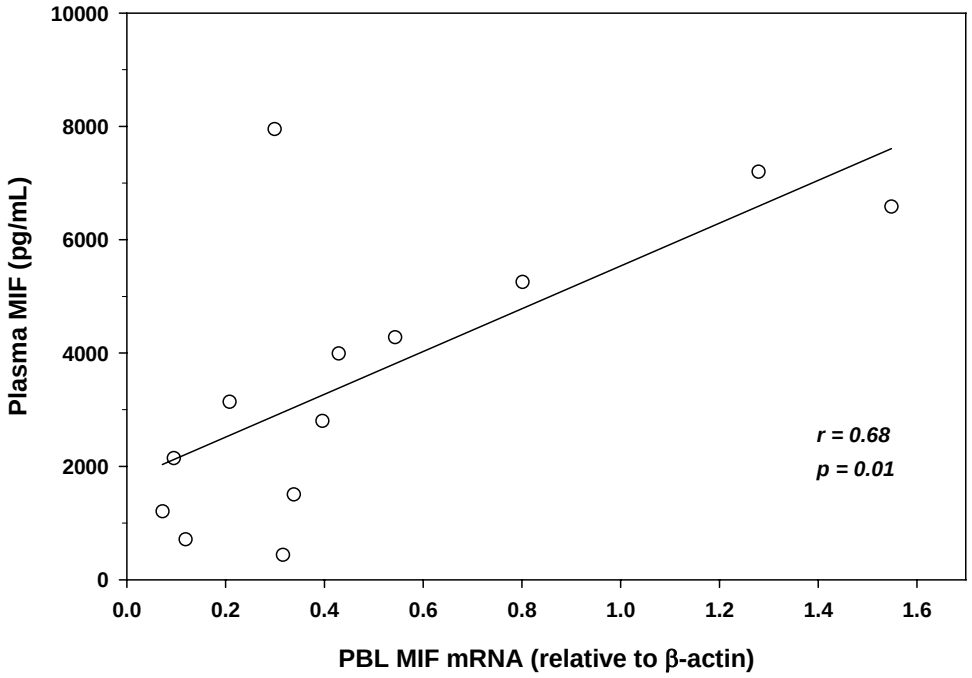



**Figure 9:** ***Correlation of peripheral blood leukocytes (PBL) MIF mRNA with circulating MIF*****.**

Levels of MIF mRNA in PBL (n=13) were determined by real time RT-PCR and expressed relative to endogenous β-actin mRNA levels. The scatter plot shows the correlation of PBL MIF mRNA with circulating MIF levels in matched samples. Statistical association was determined by Pearson's rank correlation test.

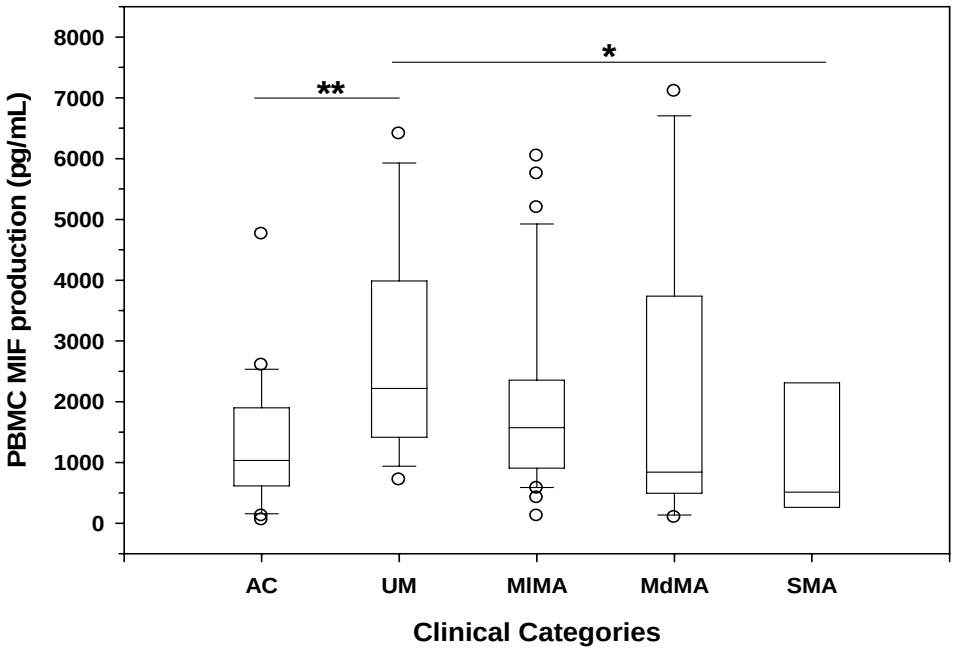
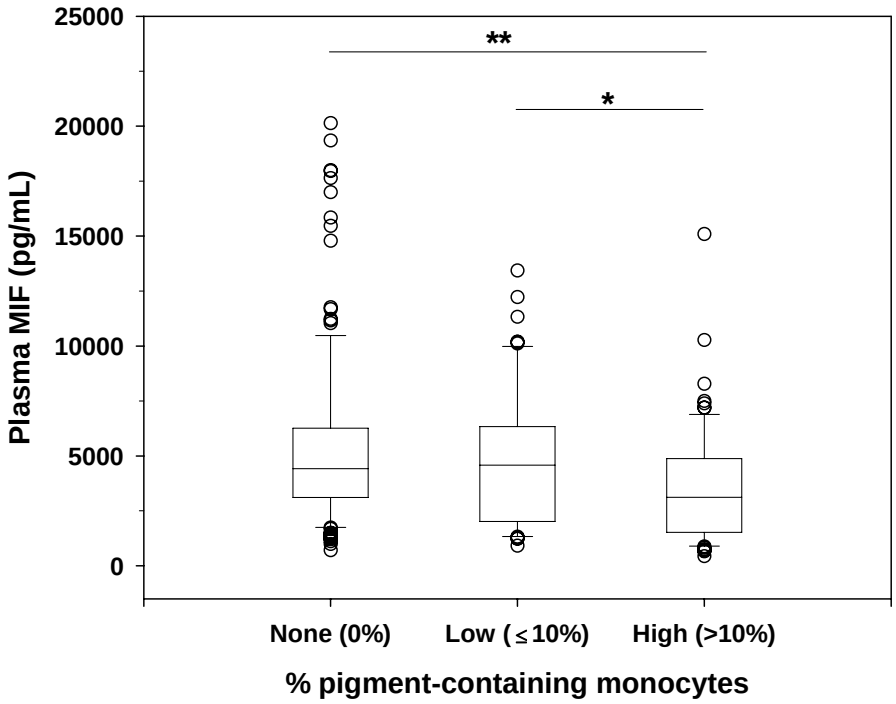



**Figure 10:** ***MIF production from peripheral blood mononuclear cells (PBMC) of children with acute malaria.***

Peripheral blood mononuclear cells (PBMC) were isolated from children with acute malaria and healthy controls (AC) and cultured for 48 hrs: AC (n=24), UM (n=15), MlMA (n=37), MdMA (n=13), and SMA (n=5). MIF concentrations in culture supernatants were determined by ELISA and presented as box plots in which the box represents the interquartile range, the line through the box is the median, whiskers show $10^{th}$ and $90^{th}$ percentiles, and symbols are outliers. Median (interquartile range) levels of MIF were: AC, 1034 (615-1902); UM, 2218 (1418-3989); MlMA, 1609 (940-2439); MdMA, 842 (495-3737); and SMA, 511 (264-2312) pg/mL). *$P$<0.05, **$P$<0.005, Mann-Whitney U tests conducted after analysis of variance (Kruskal-Wallis test) revealed significant differences across groups.

**A.**




**B.**

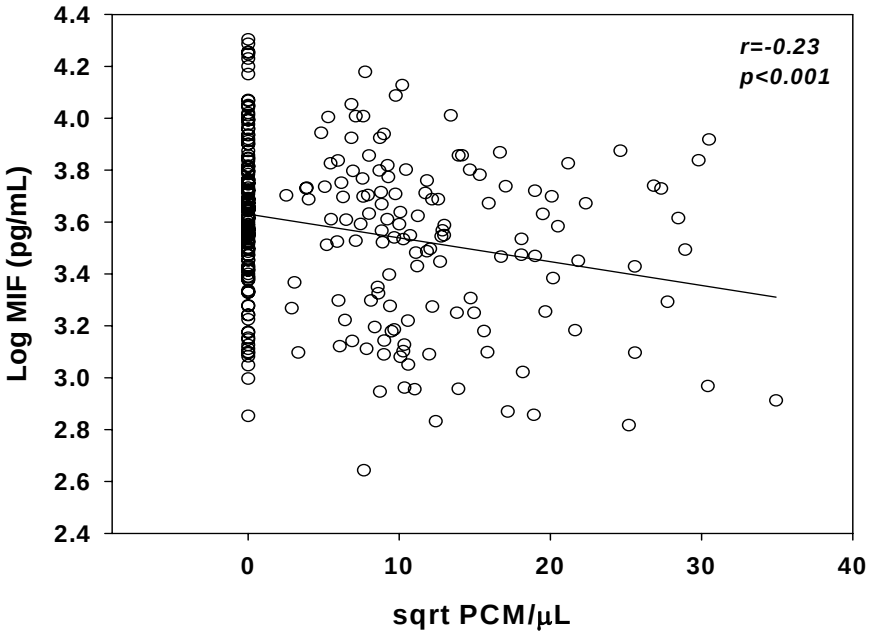
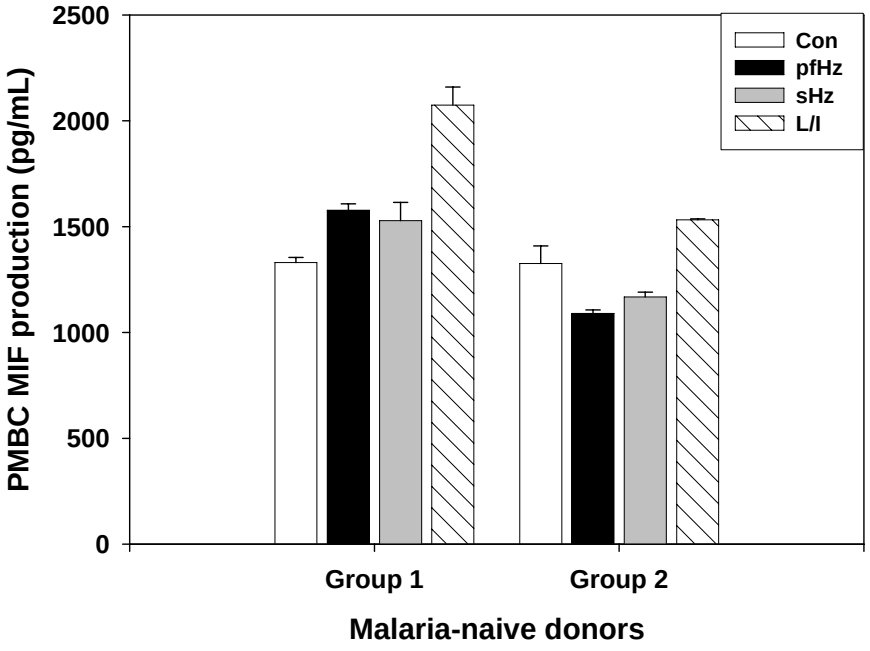



**Figure 11:** ***Relationship between plasma MIF levels and pigment-containing monocytes.***

**Figure 11.** ***Relationship between plasma MIF levels and pigment-containing monocytes.*** Giemsa-stained blood smears obtained from children with malaria were examined for the presence of pigment-containing monocytes (PCM). (**A**) Plasma levels of MIF are presented according to percentage of total monocytes containing pigment: *No PCM* (no PCM observed, n=149), *low PCM* (≤10% PCM, n=60) and *high PCM* (>10% PCM, n=79). Boxes represent the interquartile range, the line through the box is the median, whiskers show 10$^{th}$ and 90$^{th}$ percentiles, and symbols are outliers. Median (interquartile range) levels of MIF were: *No PCM*, 4417 (3112-6266); *low PCM*, 4584 (2019-6331); and *high PCM*, 4417 (3112-6266). **P*<0.05, ***P*<0.005; Mann-Whitney U tests conducted after analysis of variance (Kruskal-Wallis test) revealed significant differences across groups. (**B**) Correlation between circulating MIF concentrations and PCM/μL of blood. MIF levels were log-transformed while PCM/μL values were square-root-transformed for normality and Pearson's correlation test was used to examine statistical association.

**A.**




**B.**

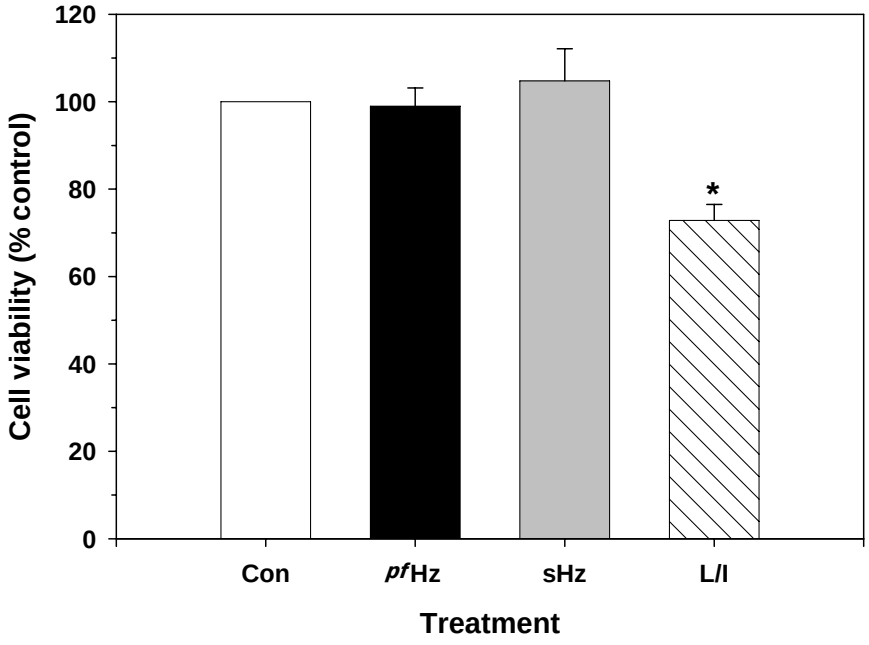



**Figure 12:** ***Effects of hemozoin on MIF production and apoptosis of cultured leukocytes.***

**C.**

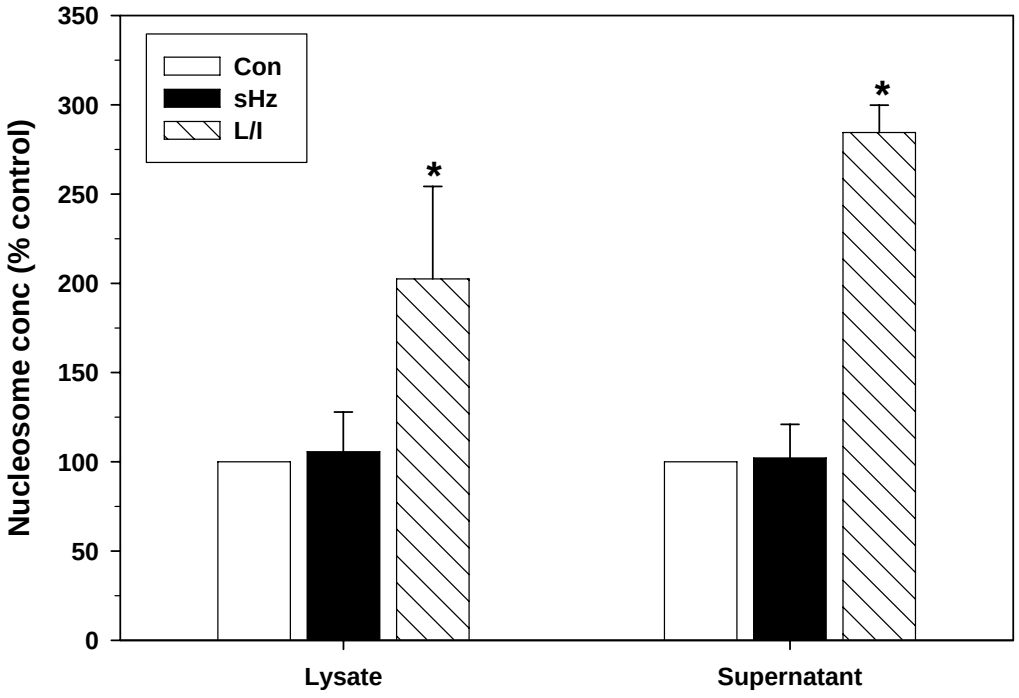



**Figure 12.** ***Effects of hemozoin on MIF production and apoptosis of cultured leukocytes***. PBMC from malaria-naïve donors were cultured ($1x10^6$ cells/mL) in the presence of media alone (Con), 10 μg/mL hemozoin (*pf*Hz), 10 μg/mL β-hematin (sHz), or a combination of 100 ng/mL lipopolysaccharide (L) and 200 U/mL IFN-γ (I). (**A**). Supernatants were harvested after 48 hrs of incubation and MIF concentrations determined by ELISA. Data shown are for three donors representative of individuals for whom MIF increased (Group 1) and three donors representative of individuals for whom MIF decreased (Group 2) in response to stimulation with *pf*Hz and sHz. Differences in MIF levels between treated cells and control conditions were statistically significant ($p<0.05$ for all comparisons, Student's t test). (**B**). Cell viability was assessed in PBMC of 3 donors after 48 hrs of culture using a methylthiazoletetrazolium (MTT) assay, and expressed as percent control. (**C**). PBMC apoptosis was determined by detecting nucleosomes in cell lysates and supernatants at 48 hrs using ELISA. Data are presented as mean (SEM) of independent experiments. **P*< 0.05 compared to Con; Student's t test.

# 4.0 CHAPTER FOUR: RESULTS, SPECIFIC AIM 2

**To investigate the role of MIF in proliferation and differentiation of erythroid progenitor cells.**

## 4.1 HYPOTHESES 1 AND 2, PRESENTATION OF MANUSCRIPT ENTITLED: *AN INVESTIGATION OF THE ROLES OF MALARIA-ASSOCIATED INFLAMMATORY MEDIATORS MACROPHAGE MIGRATION INHIBITORY FACTOR (MIF), TUMOR NECROSIS FACTOR (TNF)-α, AND NITRIC OXIDE (NO) IN REGULATION OF ERYTHROPOIESIS USING A NOVEL IN VITRO MODEL.*

*Hypothesis 1: Endogenous MIF production is required to promote efficient proliferation of erythroid progenitors.*

*Hypothesis 2: MIF-regulated inflammatory mediators TNF-α and NO are more detrimental to erythroid progenitor survival and differentiation than MIF.*

To address *hypotheses 1 and 2*, an *in vitro* model of erythropoiesis was developed and used to investigate the roles of MIF and MIF-regulated inflammatory mediators TNF-α and NO in

influencing erythroid cell development. The following manuscript describes the erythropoiesis model and contrasts the roles of MIF, TNF-α, and NO in regulating erythropoiesis.

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### 4.1.1 Footnote page

A portion of this work was presented at the 54th Annual Meeting of the American Society of Tropical Medicine and Hygiene (December 11th-15th, 2005, Washington DC, USA). The study was approved by the Ethics Committee of the University of Pittsburgh Institutional Review Board, and written, informed consent was obtained from all participants.

There is no conflict of interest for any of the authors of the manuscript due to either commercial or other affiliations.

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### 4.1.2 Abstract

One of the most lethal complications of *Plasmodium falciparum* malaria is severe malarial anemia (SMA), which appears related to malaria-induced suppression of erythropoiesis. Studies required to identify the specific mechanisms and the molecular mediators involved in inhibiting red cell development have been hampered by difficulties in obtaining bone marrows of children, and the lack of reliable models for examining erythroid development. Using different combinations of growth factors, we developed a two-step strategy for obtaining sufficient quantities of peripheral blood CD34+ stem cells in liquid culture, followed by induction of erythroid maturation in the presence of inflammatory mediators that have previously been implicated in malaria-induced suppression of erythropoiesis [i.e., macrophage migration inhibitory factor (MIF), tumor necrosis factor (TNF)-α, and nitric oxide (NO)]. In addition, since erythroid cells produce high levels of MIF endogenously, the effect of MIF-blocking antibodies on erythropoiesis was also examined. Addition of exogenous MIF and neutralization of endogenous MIF modestly increased proliferation of early erythroid progenitors, but had no noticeable effects on cellular apoptosis and maturation. However, TNF-α and NO substantially suppressed erythroid cell proliferation which appeared to be partly due to increased cellular apoptosis. In addition, NO significantly inhibited erythroid cell maturation as shown by decreased expression of the erythroid lineage marker, glycophorin A. Taken together, these results demonstrate that TNF-α and NO are more detrimental to erythroid cell development than MIF, suggesting that these mediators may play a more important role in suppressing erythropoiesis during malaria.

### 4.1.3 Introduction

Although there is convincing evidence that suppression of erythropoiesis contributes to the development of SMA (Weatherall and Abdalla 1982; Abdalla 1990; Kurtzhals *et al.* 1997; Wickramasinghe and Abdalla 2000), the precise mechanisms and molecular mediators involved are unknown. Some studies have reported inadequate production of erythropoietin (Epo) during malaria (Burgmann *et al.* 1996), however, deficient Epo production does not seem to be the major basis for inadequate erythropoiesis since appropriately increased levels of Epo in children with malarial anemia have also been reported (Burchard *et al.* 1995; Kurtzhals *et al.* 1997) (Nussenblatt *et al.* 2001). There is considerable evidence that dysregulation in innate immune responses favoring an over-production of pro-inflammatory mediators plays an important role in suppression of erythropoiesis during a malarial infection (Clark and Chaudhri 1988; Angulo and Fresno 2002; Clark and Cowden 2003; McDevitt *et al.* 2004).

Recent studies have demonstrated that MIF inhibits development of both erythroid and myeloid progenitors *in vitro*, suggesting that MIF may be a critical factor in suppression of erythropoiesis during malaria (Martiney *et al.* 2000; McDevitt *et al.* 2006). On the contrary, several other studies have described an important role for MIF in cell survival and proliferation, through activation of the MAP kinase signal cascade (Petrenko *et al.* 2003) and suppression of the pro-apoptotic factor p53 (Hudson *et al.* 1999; Mitchell *et al.* 1999; Fingerle-Rowson *et al.* 2003). In addition, MIF-knockout embryonic fibroblasts show impairment in both normal and stimuli-induced cell growth (Petrenko *et al.* 2003), in a manner analogous to the inefficient response of erythroid progenitors to increased erythropoietin in children with SMA (Burchard *et al.* 1995). Furthermore, RBCs store substantial quantities of preformed MIF (Mizue *et al.* 2000), and recent studies in our laboratory revealed that committed erythroid progenitors produce high

levels of MIF (Awandare, unpublished observations), suggesting that MIF could be required for erythroid cell development. Therefore, to evaluate the role of MIF in erythroid development, the effects of endogenously produced MIF, as well as exogenously supplemented MIF on erythropoiesis was examined. In addition, since there is over-production TNF-α and NO in children with malaria (Clark and Chaudhri 1988; Kwiatkowski *et al.* 1989; Kwiatkowski 1990; Anstey *et al.* 1999; Perkins *et al.* 2000; Keller *et al.* 2004b), the relative contributions of these inflammatory mediators in suppression of erythropoiesis will also be examined.

Studies on the mechanisms and molecular mediators involved in suppression of the erythropoietic response during human malaria have been hampered by difficulties in obtaining sufficient quantities of erythroid progenitors from bone marrows of severely anemic children and the lack of a reliable *ex vivo* or *in vitro* model for examining erythroid development. Recently, it has been shown that CD34+ hematopoietic stem cells can be cultivated in liquid culture and induced to differentiate into RBCs (Freyssinier *et al.* 1999; Neildez-Nguyen *et al.* 2002). Based on these principles, an *in vitro* model of erythropoiesis was developed and used to evaluate the effects of exogenous MIF, MIF blocking antibodies, TNF-α, and NO on erythropoiesis.

### 4.1.4 Materials and methods

#### 4.1.4.1 *Isolation of CD34+ cells.*

PBMC were isolated using Ficoll-hypaque from donor leukopack samples (50mL) obtained from the University of Pittsburgh Medical Center (UPMC) blood bank. PBMC were washed twice and resuspended in phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 0.6% anticoagulant citrate dextrose formula A (ACD-A). Hematopoietic progenitor cells (CD34+) were enriched by two cycles of positive selection using anti-CD34 microbeads and magnetic cell sorting on Midi-MACS columns (Direct CD34 Progenitor cell isolation kit, Miltenyi Biotec). The number of viable CD34+ cells obtained was determined by trypan blue exclusion.

#### 4.1.4.2 *Erythroid cell growth media.*

Culture media used in this study were based on previously described methods (Freyssinier *et al.* 1999; Neildez-Nguyen *et al.* 2002) that have been modified for selectively optimizing erythroid development with negligible contamination from other cell lineages. Basic culture medium was composed of Iscove's Modified Dulbecco's Medium (IMDM, Gibco, Invitrogen, Carlsbad, CA) containing 15% BIT (mixture of 5% BSA, 50 μg/mL bovine pancreatic insulin and 1 mg/mL human transferrin; Stem cell technology), 100 U/mL penicillin-streptomycin, and 2mM L-glutamine. Primary culture medium was prepared from the basic culture medium by adding a combination of cytokines: 10 ng/mL recombinant human (rh) IL-3, 10 ng/mL rh IL-6, and 100 ng/mL rh stem cell factor (SCF) [R&D systems, Minneapolis, MN]. Secondary cultures were

initiated by stimulation with rh erythropoietin (1 U/mL, R&D systems, Minneapolis, MN) to induce erythropoiesis.

#### 4.1.4.3 *Primary cell culture.*

CD34+ cells were plated in a 24-well plate at 1 x $10^5$ cells/well in PCM and incubated at 37 $^oC$ in a humidified atmosphere of 5% $CO_2$ and 5% oxygen for 3 days. At the end of the incubation period, cells were harvested, washed twice in IMDM and the number of viable cells determined using trypan blue exclusion.

#### 4.1.4.4 *Secondary cell culture.*

To initiate erythropoiesis, cells obtained from the primary culture were re-plated at 15 X $10^3$ cells/well and stimulated with Epo alone (Epo Control), or in the presence of rh MIF (200 ng/mL, 100 ng/mL), MIF blocking antibodies (100 μg/mL, 50 μg/mL), rh TNF-α (100 ng/mL, 50 ng/mL, BD Pharmingen), and nitric oxide donors, DETANOATE (propylamine propylamine; 100 μM, 50 μM) and PAPANOATE (diethylene triamine; 100 μM, 50 μM; Cayman Chemical), and were left to incubate as before for 11 additional days. Additional cells were cultured in the absence of Epo (No Epo) to serve as a negative control for erythropoiesis. In order to maintain optimal balance of nutrients, media were replenished every two days by replacing half of the culture medium with freshly prepared media.

Purified bioactive rMIF and mouse ascites fluid from which MIF blocking antibodies were isolated were kind gifts from Dr. Richard Bucala of Yale University, New Haven, CT. Anti-MIF monoclonal antibodies (Clone NIH3D9) were isolated from ascites fluid by Protein-A affinity purification (Pierce, Rockford IL), followed by dialysis with the Slide-A-Lyzer system (Pierce, Rockford, IL). The purified antibodies were concentrated using the

Amicon Ultra-15 centrifugal filter device (Millipore, Billerica, MA), and quantified using a Coomassie (Bradford) protein assay (Pierce, Rockford, IL) with bovine IgG as standard.

#### 4.1.4.5 *Cell proliferation assays.*

Cell proliferation was assessed using a methylthiazoletetrazolium (MTT)-based assay (Sigma, St. Louis, MO). The assay is based on the ability of viable cells to metabolize the tetrazolium salt to form an insoluble formazan dye which is then quantified by spectrophotometry. Briefly, on the indicated days, cells were resuspended by gentle tituration and 200 μL of culture from each well was transferred to a corresponding well in a 96-well plate. Twenty microliters of MTT (dissolved at 5 mg/mL in PBS) was added, and the plate was incubated under culture conditions for 4 hours. After the supernatant was removed, the formazan produced was solubilized using isopropanol and absorbance was read at 570nm. Relative cell proliferations across different conditions were determined by expressing absorbances as percentages relative to baseline (Epo Con).

#### 4.1.4.6 *Apoptosis assays.*

Cellular apoptosis was assessed by quantifying the concentrations of nucleosomes in cell lysates (early-stage apoptosis) and culture supernatants (late-stage apoptosis) using a cell death detection ELISA according to manufacturer's recommendations (Roche Diagnostics). Briefly, cells were resuspended by gentle tituration and 100 mL of culture was transferred to a 96-well plate. The plate was centrifuged at 1000 rpm for 10 mins, and the supernatants harvested for nucleosome ELISA. The cell pellet was gently washed in 100 mL of fresh PBS before lysis, followed by centrifugation and collection of lysate for nucleosome detection. To account for differences in

cell concentrations across conditions, nucleosome levels were normalized using the corresponding MTT assay data, and then expressed as fold-change relative to Epo Con.

#### 4.1.4.7 *Immunophenotypying assays.*

Maturation of erythroid cells was monitored by determination of surface expression of stage-specific markers CD34, CD45, CD71 and glycophorin A (GPA). Cells were washed and resuspended in PBS containing 0.5% BSA and incubated with fluorescein isothiocyanate (FITC) - or phycoerythrin (PE)-conjugated antibodies at $4^{o}C$ for 25 minutes. After two washes, cells were resuspended in 300 μL of PBS (containing 0.5% BSA and 1% paraformaldehyde) for flow cytometric analysis. A DNA dye, 7-amino actinomycin D (7-AAD, BD Biosciences, Franklin Lakes, NJ), was added to the stained cells 10 mins before acquisition to differentiate between dead and live cells.

### 4.1.5 Results

#### 4.1.5.1 *An in vitro model for studying regulation of erythropoiesis.*

Based on recently demonstrated techniques for cultivating hematopoietic stem cells in liquid culture (Freyssinier *et al.* 1999; Neildez-Nguyen *et al.* 2002), we developed an *in vitro* model of erythropoiesis was developed using peripheral-blood mobilized CD34+ cells. Since frequency of CD34+ cells in PBMC is ~ 0.05-0.2 %, a small quantity of CD34+ cells were first expanded without differentiation for three days using a carefully optimized cocktail of growth factors including IL-3, IL-6, and SCF (Freyssinier *et al.* 1999), and then induced towards erythroid lineage during an additional 11 days by stimulation with Epo (Figure 13). Prior to Epo stimulation, cells were divided into different culture wells and treated with the potential inhibitors under investigation. The efficiency and effectiveness of erythropoiesis was continuously monitored during 14 days of erythroid cell growth and development by examining cell proliferation and differentiation (maturation) (Figure 13). Cell proliferation was measured using a biochemical method, which quantifies cellular metabolism, in place of the traditional methods such as microscopical enumeration of cells. In addition, since there is differential expression of surface markers at key developmental stages of erythroid cell maturation (Figure 14), cell differentiation during secondary culture was monitored using immunophenotypic analyses by multi-color flow cytometry. CD34+ cells develop through the burst-forming units-erythoid (BFU-E) and colony-forming unit-erythroid (CFU-E) stages to more mature erythroblasts and reticulocytes by losing CD34 and CD45 expression, while gaining expression of CD71 (transferrin receptor) and glycophorin-A (GPA). GPA is an anchor protein expressed

exclusively on the surface of mature erythroid lineage cells, and is thus a reliable marker of erythroid cell development (Xiao *et al.* 2002).

Relative to unstimulated cells (No Epo), there was rapid proliferation of erythroid cells in response to Epo stimulation during 11 days of secondary culture (Figure 15A). The immunophenotypic scheme for analyzing erythroid cell differentiation in response to Epo is illustrated in Figure 15B. Immature CD34+ progenitors on day 3 express high levels CD45 and CD71, but are negative for GPA. By day 10 (7 days of Epo stimulation), expression of CD34 is completely lost and CD45 is down-regulated, with a majority of cells expressing GPA, demonstrating erythroid maturation (Figure 15B, and Table 5). Of note, expression of other lineage markers, including CD3, CD14, and HLA-DR was very low or absent (Table 5, and data not shown), confirming that the vast majority of the cells were committed erythroid cells by day 10. On the contrary, cells cultured in the absence of Epo (No Epo) retained high expression of CD34 and CD45, expressed low levels of GPA, and also expressed HLA-DR (Table 5).

**4.1.5.2** ***Effects of MIF, TNF-α and NO on erythroid cell proliferation during erythropoiesis.***

Although previous studies demonstrated that MIF suppressed hematopoiesis, studies in our laboratory show that erythroid progenitors produced high levels of MIF during erythropoiesis (Figure 15C). To examine the role of MIF in erythroid cell development, erythropoiesis was induced in the presence of either exogenous MIF (rMIF; 200 and 100 ng/mL), at doses that suppressed hematopoiesis in previous studies (McDevitt *et al.* 2006), or MIF blocking antibodies (anti-MIF; 100 and 50 μg/mL), at concentrations sufficient to completely block endogenous MIF (Calandra *et al.* 2000). In addition, since there is over-production of TNF-α and NO in children with malaria, the effects of these other malaria-associated inflammatory mediators on

suppression of erythropoiesis were also examined. NO was provided in culture by two NO donors: one with a very short half-life (PAPANONOATE, 15 mins), and another with a longer half-life (DETANONOATE, 22 hrs). Addition of rMIF at 200 and 100 ng/mL marginally increased erythroid cell proliferation on days 6 and 8 of culture ($P<0.05$ for all comparisons) relative to baseline levels, however, by day 14 cultures both doses of rMIF significantly decreased cellular proliferation ($P<0.05$, Figure 16A). Interestingly, treatment with anti-MIF increased proliferation on days 6 and 8 ($P<0.05$ relative to baseline, and isotype control treatment), but this effect disappeared by day 10, with cell numbers being similar to baseline levels by day 14 (Figure 16B). On the contrary, TNF-α (100 and 10 ng/mL) exerted a substantial and sustained, dose-dependent suppressive effect on erythroid cell proliferation throughout the culture period, and these effects where statistically significant on days 8, 10 and 14 ($P<0.05$ for all comparisons, Figure 16C). Treatment with both PAPANONOATE (PAPANO, 100 and 50 μM) and DETANONOATE (DETANO, 100 and 50 μM) elicited similar patterns of cell proliferation, characterized by a marked early, dose-dependent suppression on days 6 and 8 ($P<0.05$ for all comparisons, Figures 16D and 16E). Although there was a trend towards recovery by days 10 and 14, cell proliferation in the presence of NO donors remained below baseline levels, maintaining statistically significant differences for DETANO ($P<0.05$ for all comparisons, Figure 16D), but not for PAPANO ($P<0.10$ for all comparisons, Figure 16E). These results demonstrate that TNF-α and NO are much more detrimental to erythroid cell development than MIF. In addition, endogenously produced MIF does not appear to play a critical role in erythropoiesis.

#### 4.1.5.3 *Effects of MIF, TNF-α and NO on erythroid cell survival during erythropoiesis.*

To investigate whether the effects of MIF, TNF-α, and NO on erythroid cell proliferation was associated with changes in cell survivability, cellular apoptosis was determined by measuring the release of nucleosomes. To ensure detection of total apoptosis, nucleosome concentrations were determined in both cell lysates (early-stage apoptosis), and culture supernatants (late-stage apoptosis) after 3 days of stimulation. Although rMIF marginally increased apoptosis of erythroid cells, these effects were not statistically significant ($P<0.10$ for all comparisons, Figure 17A). In addition, cellular apoptosis in the presence of anti-MIF was indistinguishable from levels observed at baseline and in IgG-treated cells ($P>0.50$ for all comparisons, Figure 17A). Similarly, TNF-α had no significant effects on erythroid cell apoptosis relative to baseline levels ($P>0.05$ for all comparisons, Figure 17B). Late-stage apoptosis was increased in cells cultured in the presence of PAPANO, and this difference was statistically significant for the higher dose of PAPANO ($P<0.05$), but not for the lower dose ($P=0.2$, Figure 17C). PAPANO had no significant effect on levels of early-stage apoptosis ($P>0.5$ for all comparisons, Figure 17C). There was a significant and dose-dependent increase in erythroid cell apoptosis in the presence of DETANO ($P<0.05$ for all comparisons), with the differences being more pronounced for late-stage apoptosis (Figure 17C). Taken together, these data demonstrate that, except for TNF-α, suppression of erythroid cell proliferation is generally associated with an increased level of apoptosis.

#### 4.1.5.4 *Effects of MIF, TNF-α and NO on differentiation of erythroid progenitors in response to Epo stimulation.*

Suppression of erythropoiesis during malaria results from both ineffective reticulocyte production and dyserythropoiesis (Wickramasinghe and Abdalla 2000; Chang and Stevenson 2004), suggesting that erythroid cell maturation may be impaired. Therefore, the roles of MIF, TNF-α and NO in regulating erythroid cell differentiation in response to Epo stimulation was investigated by examining expression of CD34, CD45, CD71 and GPA (Table 5 and Figure 18). There were no significant differences in expression of maturation markers between cells treated with rMIF or anti-MIF and control conditions (Table 5, and Figures 18A and 18B). Treatment with a high dose of TNF-α seemed to slightly augment erythroid cell maturation as evidenced by an accelerated down-regulation of CD45 and a marginal increase in GPA expression; however, these differences were not statistically significant ($P>0.5$ for all comparisons; Table 5 and Figure 18C). Conversely, in the presence high doses (100 μM) of either NO donors, there was higher retention of CD45 and CD34, and less GPA expression relative to Epo Con (Table 5, Figure 18D and 18E), indicating an inhibition of erythroid cell maturation. These effects of NO were dose-dependent, and reached statistical significance for DETANO ($P<0.05$ for all comparisons) but not PAPANO ($P>0.1$ for all comparisons). Thus, while MIF and TNF-α did not appear to influence erythroid cell differentiation, sustained NO stimulation markedly suppresses maturation of erythroid progenitors during erythropoiesis.

### 4.1.6 Discussion

Although previous studies have implicated MIF in the suppression of erythropoiesis during murine malaria (Martiney *et al.* 2000; McDevitt *et al.* 2006), results presented in Specific Aim 1 demonstrate that peripheral blood MIF production is not significantly related to reticulocyte production in children with malarial anemia. Therefore, it was of interest to further define the role of MIF in regulating erythropoiesis, and to examine the potential contribution of MIF relative to other malaria-associated inflammatory mediators in inhibiting erythroid cell development. However, a major impediment to obtaining a complete understanding of the mechanisms contributing to suppression of erythropoiesis during malaria is the difficulty associated with obtaining bone marrow samples from severely anemic children, coupled with the lack of a reliable *in vitro* model for studying the regulation of erythropoiesis. Studies presented here describe a novel *in vitro* erythropoiesis model using peripheral blood mobilized CD34+ hematopoietic stem cells cultivated in liquid culture. Our results show that a small number of CD34+ cells can be successfully amplified with growth factors to sufficient numbers for testing soluble mediators of inflammation that may alter erythropoiesis, avoiding the requirement for bone marrow cells. Furthermore, the use of molecular/biochemical methods for assessing cell proliferation and apoptosis, and flow cytometry for phenotypic characterization of erythroid cells, provides a more reliable and objective alternative to exclusive use of microscopic analysis. The method developed here showed reliability and reproducibility in comparing the effects of the potential inhibitors of erythroid development investigated.

Contrary to previous studies demonstrating that MIF inhibited the development of hematopoietic cells (Martiney *et al.* 2000; McDevitt *et al.* 2006), results presented here show a

bi-phasic effect of MIF on erythropoiesis: slightly augmenting proliferation of early progenitors, and marginally suppressing growth of older cells. In addition, since MIF is ubiquitous, we tested erythroid cell culture supernatants for MIF protein as part of our investigations. These analyses showed, for the first time, that erythroid progenitors produced large quantities of MIF during erythropoiesis, raising the possibility of a positive role for MIF in erythropoiesis. However, neutralization of endogenous MIF using anti-MIF blocking antibodies did not reveal any adverse effects on erythropoiesis; instead it slightly augmented early erythroid cell proliferation. Although the similar effects elicited by both rMIF and anti-MIF appear contradictory, these results are consistent with the fact that various studies have described both pro- and anti-proliferative properties of MIF (Martiney *et al.* 2000; McDevitt *et al.* 2006; Hudson *et al.* 1999; Mitchell *et al.* 1999; Fingerle-Rowson *et al.* 2003; Petrenko *et al.* 2003), suggesting that MIF may be important in maintaining a balanced erythropoietic response.

Since several inflammatory mediators are over-produced during malaria, it was of interest to investigate the potential roles of MIF-regulated mediators in contributing to suppression of erythropoiesis. Of the comprehensive panel of mediators examined in *Specific Aim 1*, TNF-α and NO oxide were the most-upregulated pro-inflammatory mediators in children with acute malaria. Since previous studies demonstrate that TNF-α and NO can suppress erythroid cell development *in vivo* and *in vitro* (Johnson *et al.* 1989; Rusten and Jacobsen 1995; Shami and Weinberg 1996; Anstey *et al.* 1999; Xiao *et al.* 2002), the effects of these mediators on erythropoiesis were examined along with MIF. Unlike the modest effects observed for MIF, treatment with both TNF-α and NO substantially suppressed erythroid cell proliferation, with inhibition margins of up to 65% for TNF-α, and 90% for NO. Furthermore, while MIF and TNF-α had no significant effects on erythroid cell differentiation, high levels of NO significantly

inhibited maturation of erythroid cells, characterized by reduced GPA expression. While the mechanisms by which TNF-α inhibited erythropoiesis in the model system are not clear, measurement of cellular apoptosis revealed that NO-induced suppression of erythropoiesis is, at least in part, due to induction of cell death. Taken together, results presented here demonstrate that TNF-α and NO, rather than MIF, may be the major suppressors of erythropoiesis in children with malarial anemia.

**Table 5:** ***Phenotypic characterization of erythroid cell maturation status on day 10***

| **Conditions** | **Mean (SEM) percent of total live cells expressing markers** | | | |
|---|---|---|---|---|
| | ***GPA*** | ***CD45*** | ***CD34*** | ***HLA-DR*** |
| Epo Con | 87 (4) | 21 (2) | 1 (0) | 0 (0) |
| rMIF (200 ng/mL) | 91 (0) | 30 (0) | 0 (0) | 0 (0) |
| rMIF (100 ng/mL) | 91 (0) | 27 (0) | 0 (0) | 0 (0) |
| IgG control (100 μg/mL) | 92 (0) | 28 (1) | 0 (0) | 0 (0) |
| Anti-MIF (100 μg/mL) | 91 (1) | 27 (0) | 0 (0) | 0 (0) |
| Anti-MIF (50 μg/mL) | 91 (1) | 29 (1) | 0 (0) | 0 (0) |
| PAPANO (100 μM) | 83 (6) | 20 (4) | 2 (1) | 0 (0) |
| PAPANO (50 μM) | 81(6) | 25 (3) | 2 (0) | 0 (0) |
| DETANO (100 μM) | 70 (4) | 37 (2) | 11 (1) | 0 (0) |
| DETANO (50 μM) | 76 (4) | 29 (2) | 5 (1) | 0 (0) |
| TNF-α (100 ng/mL) | 87 (6) | 14 (5) | 1 (0) | 1 (0) |
| TNF-α 10 (10 ng/mL) | 87 (6) | 16 (5) | 1 (0) | 1 (0) |
| No Epo | 16 (5) | 92 (2) | 44 (3) | 44 (3) |

Data shown are proportions of gated live (7-AAD-) cells that were positive for the indicated surface markers, and are presented as mean (SEM) for 2 to 4 experiments.

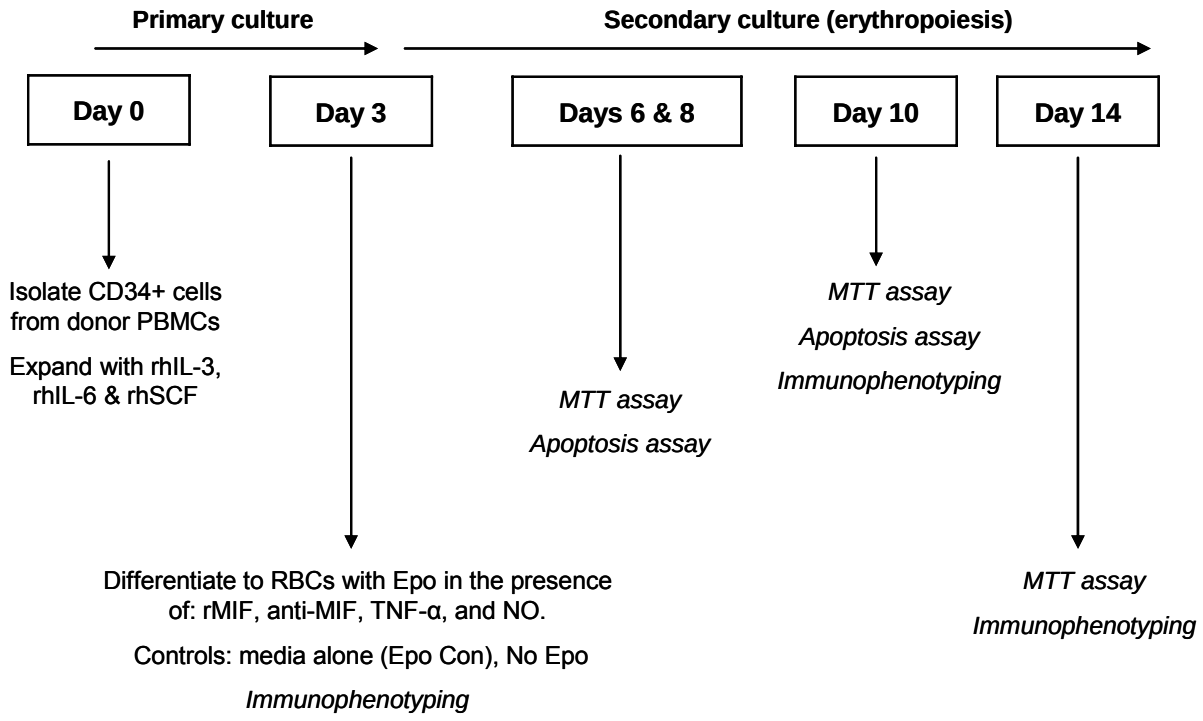



**Figure 13:** ***Experimental design for in vitro model of erythropoiesis.***

CD34+ hematopoietic stem cells were isolated from donor PBMC by labeling them with magnetic bead-conjugated anti-CD34 monoclonal antibodies followed by positive selection on a magnetic column. Purified CD34+ cells were expanded for 3 days in stem cell growth media containing IL-3, IL-6, and SCF. After 3 days of primary culture, cells were induced toward erythroid lineage development by the addition of erythropoietin alone (Epo Con), or in the presence of rMIF, anti-MIF, TNF-α, or NO. During 11 days of erythropoiesis, cell proliferation (MTT assay), apoptosis, and maturation (immunophenotyping by flow cytometry) were measured at the indicated time points.

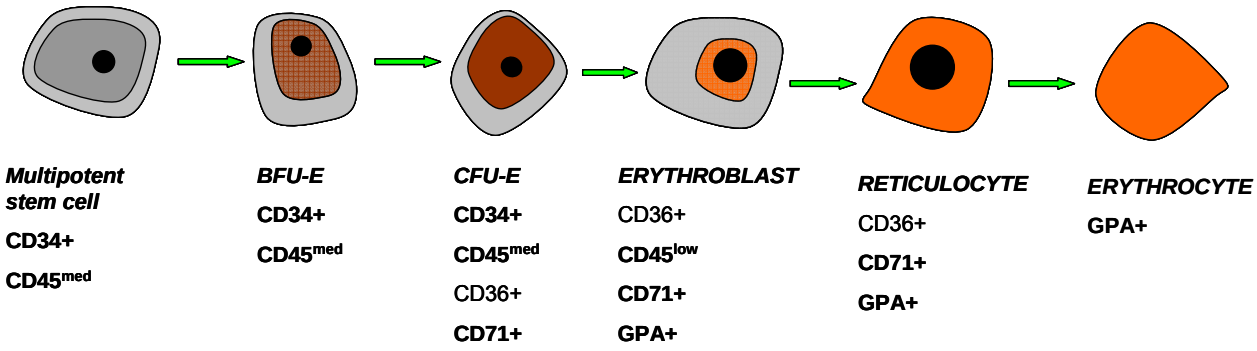
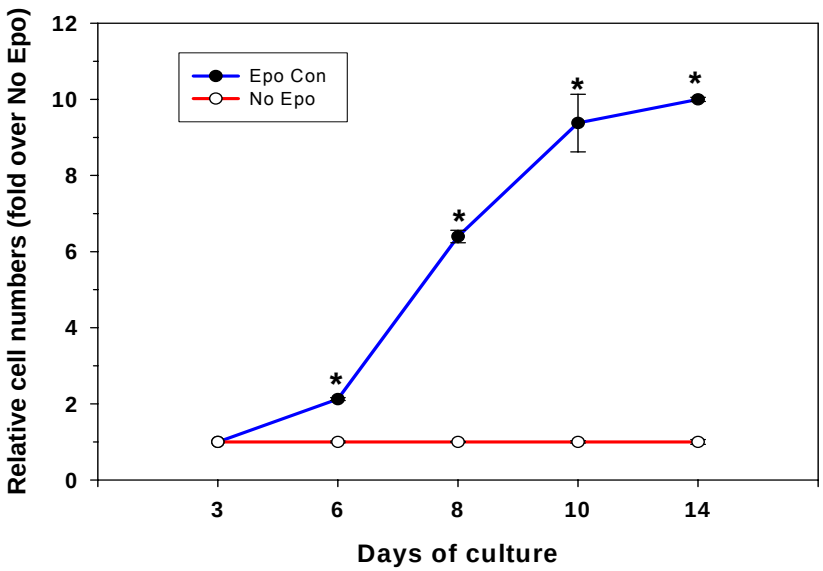



**Figure 14:** ***Phenotypic markers expressed at key developmental stages of erythroid lineage cells.***

In response to signals from erythropoietin (Epo), multipotent CD34+ stem cells commit to the erythroid lineage, forming burst-forming units (BFU-E). BFU-Es develop into colony-forming units (CFU-E) with the appearance of surface markers including CD71 and CD36. Under appropriate conditions, including sufficient Epo signals, CFU-Es mature into glycophorin A (GPA)-expressing erythroblasts, accompanied by loss of CD34 and down-regulation of CD45. Following active hemoglobin synthesis, erythroblasts develop into reticulocytes, which mature into erythrocytes accompanied by enucleation and loss of CD71 expression. Markers selected for immunophenotyping are indicated in bold font.

A.




B.

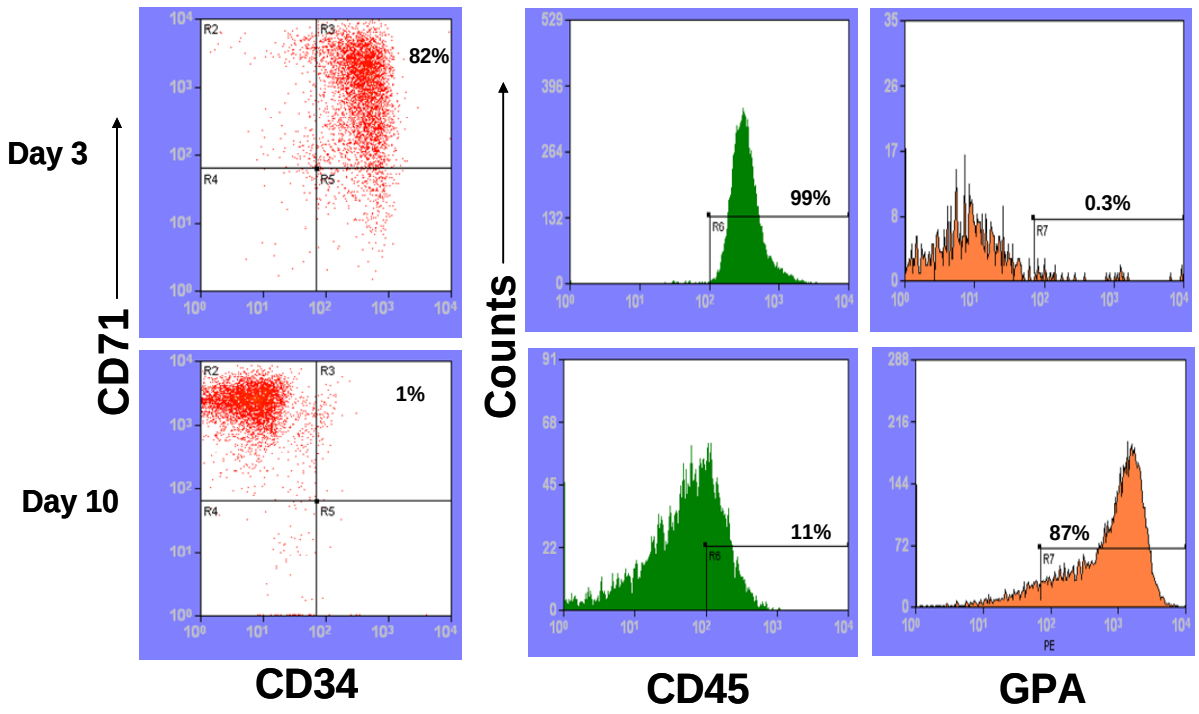



**Figure 15:** ***Proliferation, differentiation, and MIF production of erythroid progenitors.***

**C.**

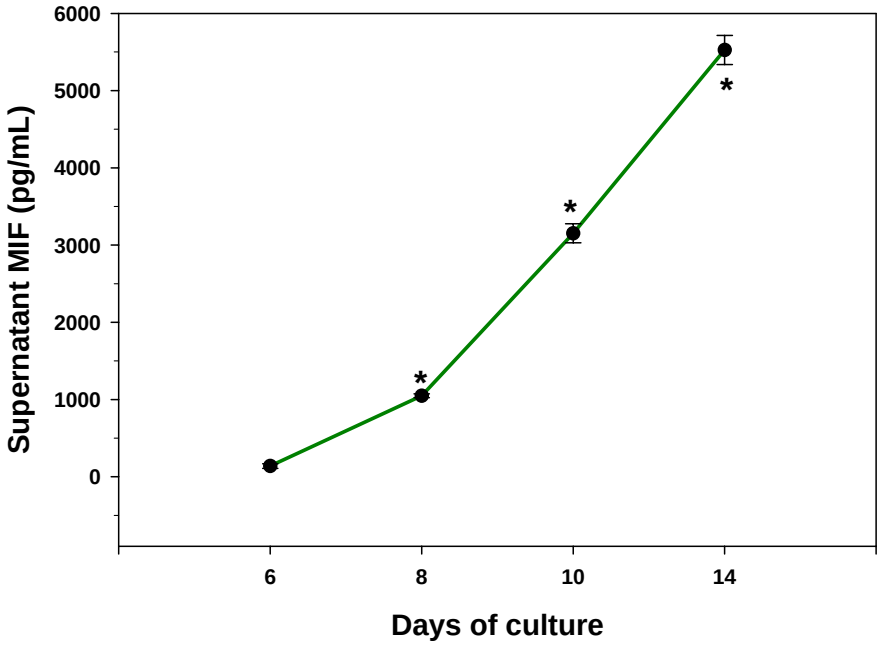
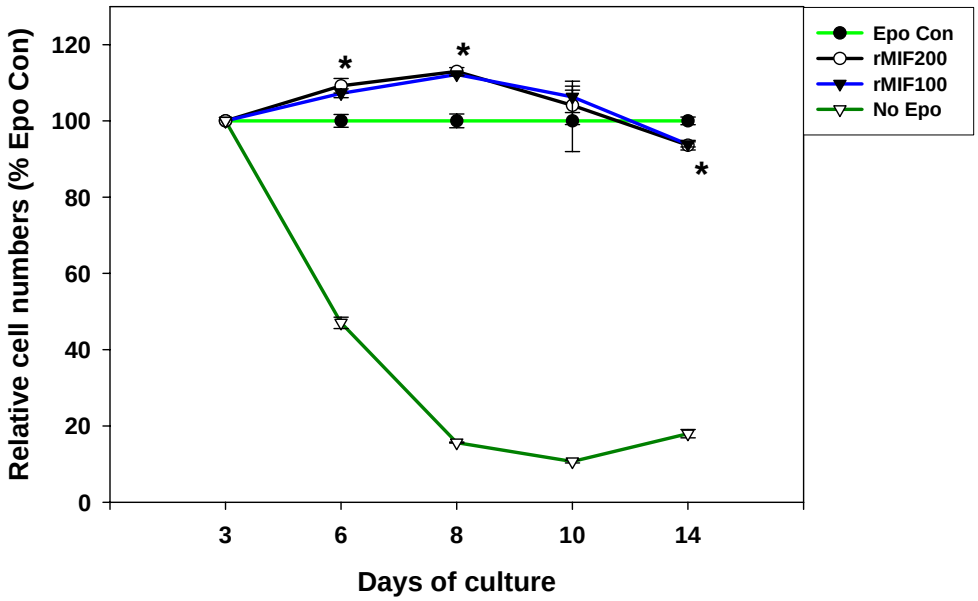



**Figure 15:** ***Proliferation, differentiation, and MIF production of erythroid progenitors during erythropoiesis.*** Peripheral blood-mobilized CD34+ stem cells were expanded in liquid culture for 3 days using a combination of growth factors (including IL-6, IL-3 and SCF), and then induced towards erythroid lineage by the addition of Epo for a further 11 days. **A)** Cell proliferation in response to Epo (Epo Con) was measured by the MTT assay and expressed as fold-change relative to cells cultured without Epo (No Epo). Data show means (SEM) for 5 independent experiments. *$P$<0.05 relative to day 3. **B)** Erythroid cell maturation during erythropoiesis was analyzed by immunophenotypic characterization. Surface expression of CD34, CD71, CD45 and glycophorin A (GPA) was determined by staining with FITC- or PE-conjugated antibodies and analyzed by flow cytometry. Data show expression of surface markers in gated live cells (7AAD-) before addition of Epo (Day 3) and 7 days after initiation of Epo stimulation (Day 10). Percentages shown are the proportion of total events within the indicated region. **C)** Supernatants were harvested from erythroid cultures at the indicated time points and analyzed for MIF protein levels using ELISA. Data show means (SEM) of two independent experiments set up in duplicate. *$P$<0.05 relative to day 6.

**A.**




**B.**

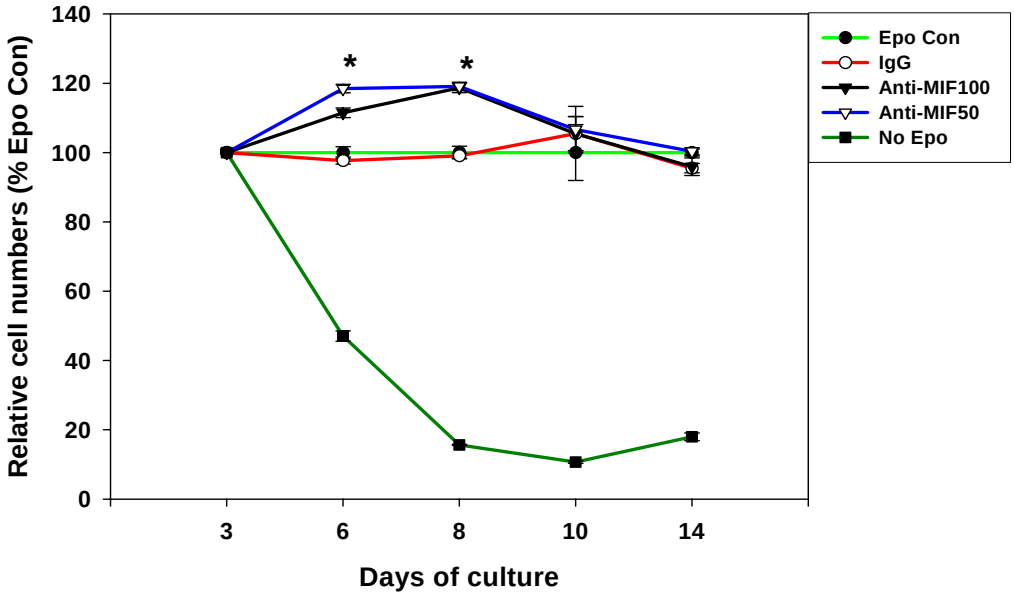
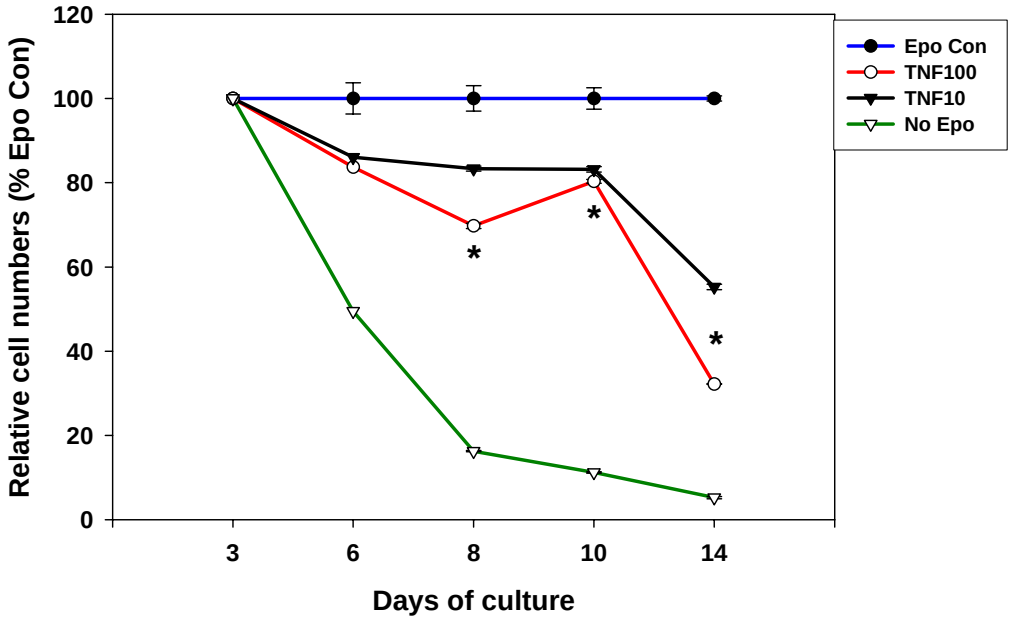



**Figure 16***: Effects of rMIF, anti-MIF, TNF-α and NO on proliferation of erythroid cells*

**C.**




**D.**

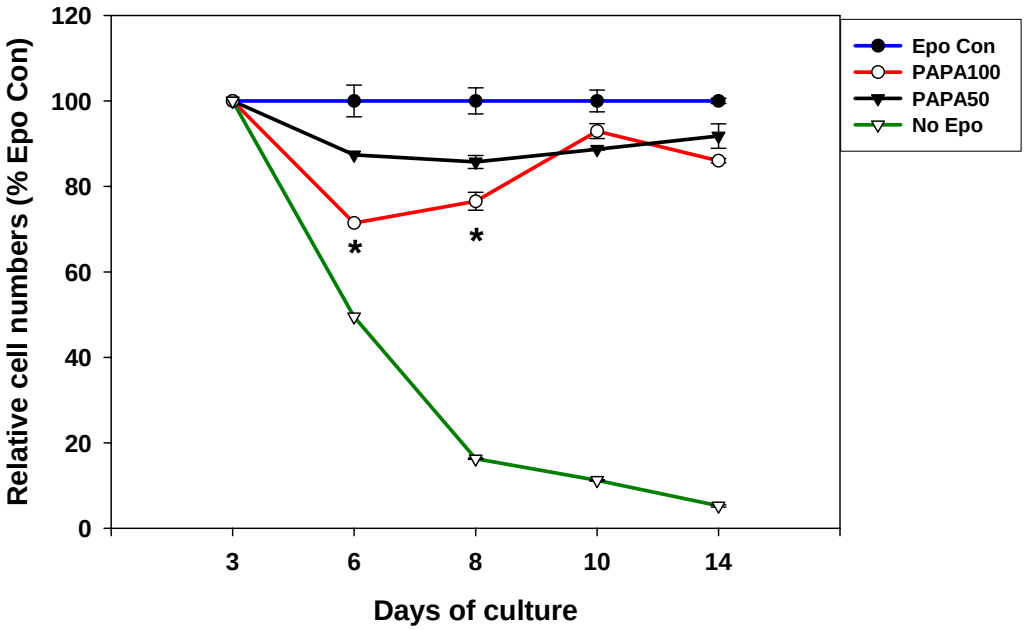



**Figure 16***: Effects of rMIF, anti-MIF, TNF-α and NO on proliferation of erythroid cells*

**E.**

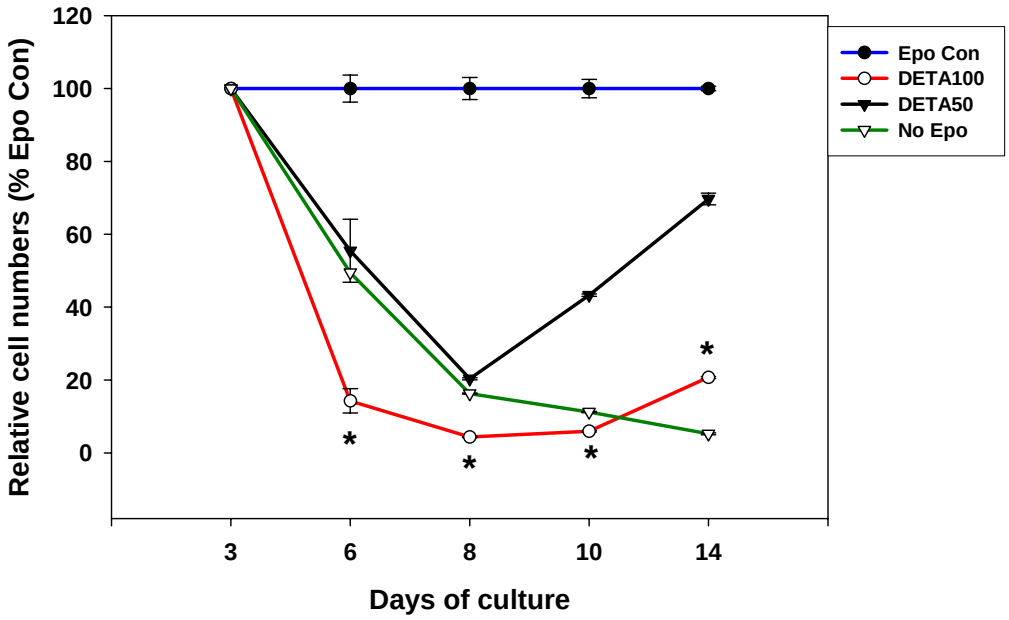
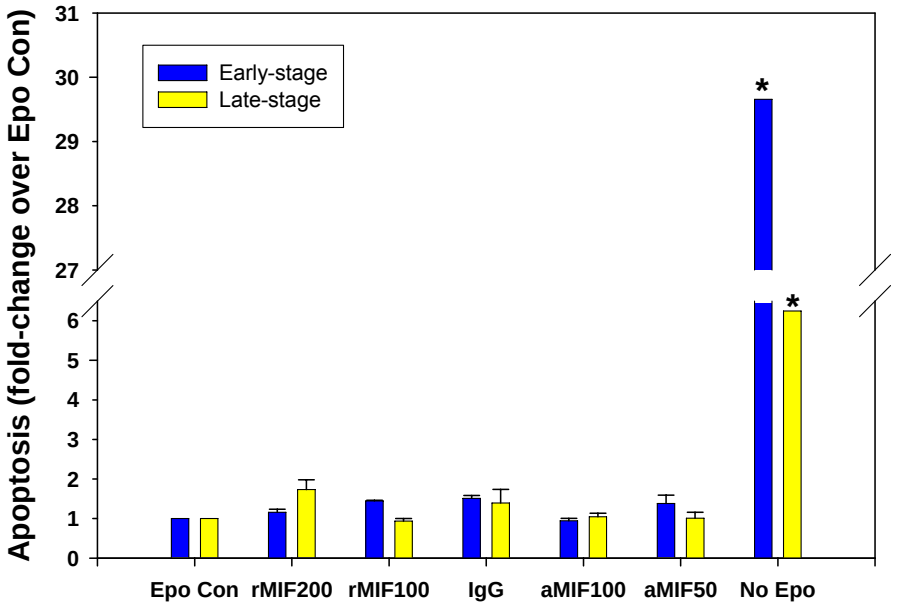



**Figure 16***: Effects of rMIF, anti-MIF, TNF-α and NO on proliferation of erythroid cells.* CD34+ stem cells were induced to undergo erythropoiesis by erythropoietin (Epo) stimulation alone (Epo Con), or in the presence of: **A)** rMIF (200 and 100 ng/mL), **B)** anti-MIF blocking antibodies (100 and 50 μg/mL), or IgG isotype control antibody (100 μg/mL); **C)** TNF-α (100 and 10 ng/mL), **D)** nitric oxide (NO) donor, PAPANONOATE (100 and 50 μM), and **E)** NO donor, DETANOATE (100 and 50 μM). As an additional control, some cells were cultured without Epo (No Epo). Cell proliferation was measured at the indicated days using a methylthiazoletetrazolium (MTT)-based assay. Data show cell proliferation of erythroid cells expressed relative to baseline conditions (Epo Con), and are presented as mean ± SEM of 5 independent experiments. *Indicate time points at which treatments with both doses of the mediators had statistically significant effects relative to Epo Con ($P<0.05$, paired t test).

**A.**




**B.**

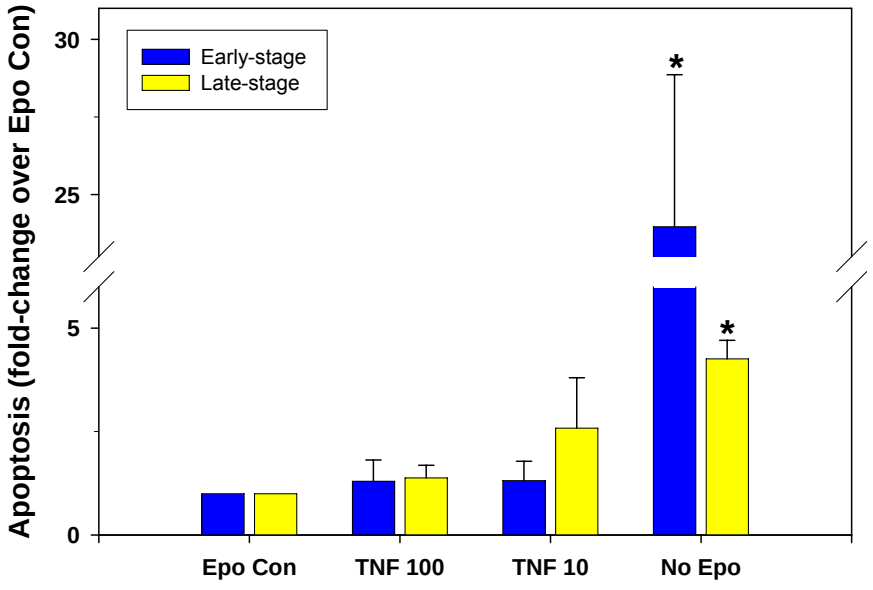



**Figure 17***: Effects of rMIF, anti-MIF, TNF-α and NO on survival of erythroid cells*

**C.**

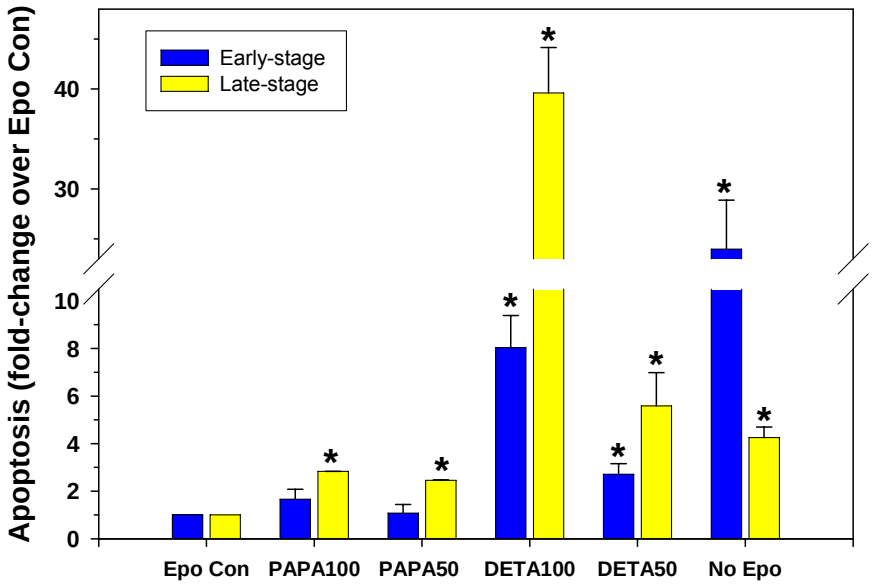



**Figure 17*: Effects of rMIF, anti-MIF, TNF-α and NO on survival of erythroid cells.***

CD34+ stem cells were induced to undergo erythropoiesis by erythropoietin (Epo) stimulation alone (Epo Con), or in the presence of: **A)** rMIF (200 and 100 ng/mL), anti-MIF blocking antibodies (100 and 50 μg/mL), or IgG isotype control antibody (100 μg/mL); **B)** TNF-α (100 and 10 ng/mL), and **C)** nitric oxide (NO) donors, PAPANONOATE (100 and 50μM), and DETANOATE (100 and 50μM). As an additional control, some cells were cultured without Epo (No Epo). Cellular apoptosis was examined after 3 days of stimulation by measuring the release of nucleosomes in cell lysates (early-stage apoptosis), and supernatants (late-stage apoptosis) by ELISA. Nucleosome concentrations were expressed as fold-change relative to baseline conditions (Epo Con), and are presented as mean (SEM) of 3 independent experiments. *Indicate treatments that had statistically significant effects relative to Epo Con ($P<0.05$, paired t test).

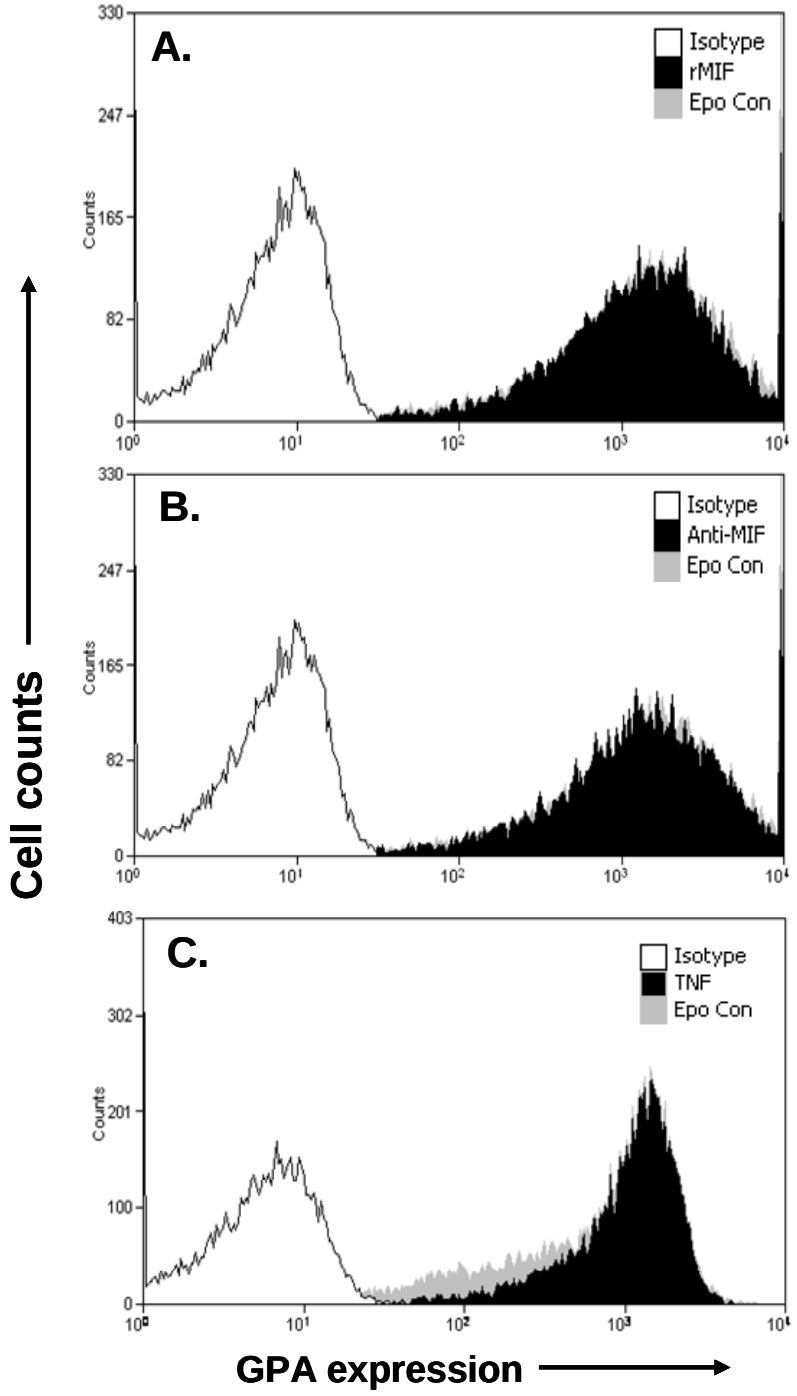



**Figure 18:** ***Effects of rMIF, anti-MIF, TNF-α and NO on erythroid cell maturation.***

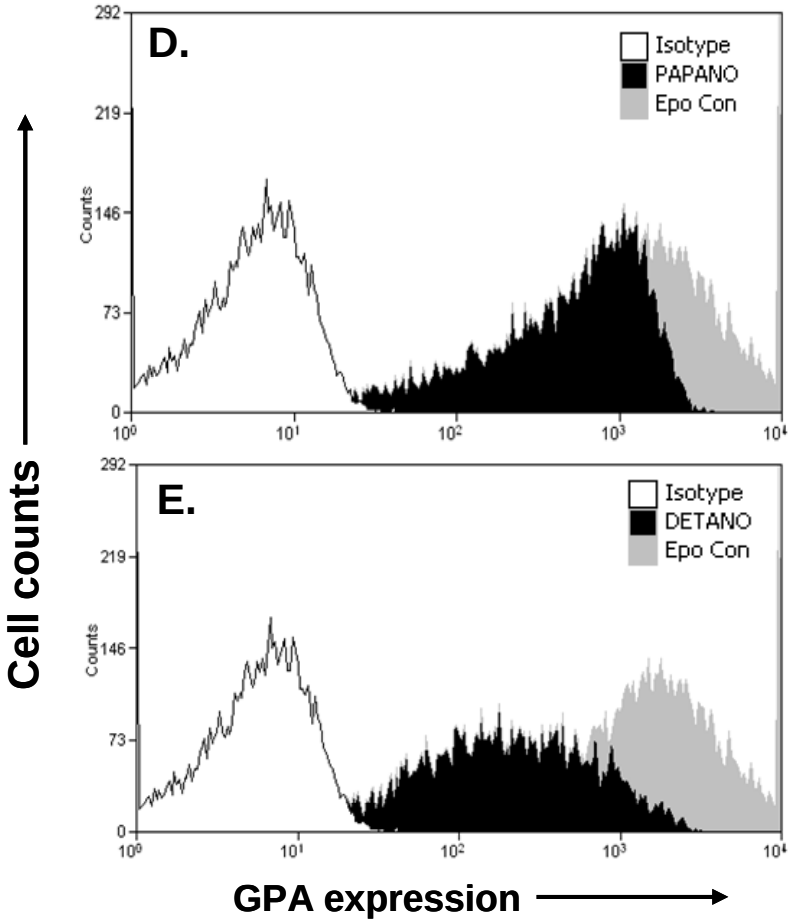



**Figure 18***: Effects of rMIF, anti-MIF, TNF-α and NO on survival of erythroid cells.*

CD34+ stem cells were induced toward erythroid differentiation by erythropoietin (Epo) stimulation alone (Epo Con), or in the presence of: **A)** rMIF (200 ng/mL), **B)** anti-MIF blocking antibodies (100 μg/mL), **C)** TNF-α (100 ng/mL), **D)** nitric oxide (NO) donor, PAPANONOATE (100 μM), and **E)** NO donor, DETANOATE (100 μM). Erythroid cell maturation was analyzed by determining surface expression of the erythroid lineage-specific marker glycophorin-A (GPA). Histogram overlays show changes in GPA expression elicited by treatment with high doses of the respective mediators relative to baseline control conditions (Epo Con) on Day 10 of culture. Data are shown for gated live cells (7-AAD-), and cell counts are normalized for each histogram. Data are representative of 2 to 4 experiments.

# 5.0 CHAPTER FIVE: RESULTS, SPECIFIC AIM 3

**To examine the influence of genetic variation at positions -173 (G/C) and -794 ($CATT_{5-8}$) of the MIF promoter on MIF production and susceptibility to SMA in children.**

## 5.1 HYPOTHESIS 1, PRESENTATION OF MANUSCRIPT ENTITLED: *A MACROPHAGE MIGRATION INHIBITORY FACTOR PROMOTER POLYMORPHISM IS ASSOCIATED WITH HIGH-DENSITY PARASITEMIA IN CHILDREN WITH MALARIA*.

*Hypothesis 1: MIF -173 and -794 variants that are associated with decreased MIF production also condition increased susceptibility to SMA.*

To address one part of *hypothesis 1*, the MIF -173 polymorphism was analyzed in the cohort of Kenyan children enrolled for the investigations in *Specific Aim 1*. The role of variation at MIF -173 in conditioning susceptibility to severe malaria (defined as HDP and/or SMA) in children residing in an area with holoendemic *P. falciparum* transmission was examined. In addition, the functional significance of the MIF -173 SNP in influencing MIF production was investigated. The findings of these investigations are presented in the following manuscript published in the journal *Genes and Immunity* (Gene Immun 2006, 7(7):568-75; reproduced with permission from Nature Publishing Group).

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**Word counts:**

Abstract: 200

Text: 3492

### 5.1.1 Footnote page

The study was approved by the Ethics Committee of the Kenya Medical Research Institute (KEMRI) and the University of Pittsburgh Institutional Review Board. Written informed consent was obtained from the parents/legal guardians of all participating children.

There is no conflict of interest for any of the authors of the manuscript due to either commercial or other affiliations.

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### 5.1.2 Abstract

Macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine that regulates innate and adaptive immune responses to bacterial and parasitic infections. Functional promoter variants in the MIF gene influence susceptibility to inflammatory diseases in Caucasians. Since the role of genetic variation in the MIF gene in conditioning malaria disease outcomes is largely unexplored, the relationship between a G to C transition at MIF -173 and susceptibility to high-density parasitemia (HDP) and severe malarial anemia (SMA) was examined in Kenyan children (aged 3-36 mos; n=477) in a holoendemic *Plasmodium falciparum* transmission region. In a multivariate model, controlling for age, gender, HIV-1 status, and sickle-cell trait, MIF -173CC was associated with an increased risk of HDP compared to MIF -173GG. No significant associations were found between MIF -173 genotypic variants and susceptibility to SMA. Additional studies demonstrated that homozygous G alleles were associated with lower basal circulating MIF levels relative to the GC group. However, stimulation of cultured peripheral blood mononuclear cells with malarial pigment (hemozoin) increased MIF production in the GG group and decreased MIF production in the GC group. Thus, variability at MIF -173 is associated with functional changes in MIF production and susceptibility to HDP in children with malaria.

**Running title:** MIF -173 SNP associated with malaria parasitemia

**Key words:** Macrophage migration inhibitory factor (MIF), genetic polymorphism, parasitemia, severe malaria

### 5.1.3 Introduction

Malaria is one of the leading causes of childhood morbidity and mortality in sub-Saharan Africa, accounting for 25-35% of the outpatient visits, 20-45% of the hospital admissions, and up to 35% of inpatient deaths (WHO 2005). The vast majority of the global malaria cases occur in sub-Saharan Africa in which greater than 90% of the clinical cases are caused by *Plasmodium falciparum* infections (WHO 2005). Clinical manifestations of *P. falciparum* malaria vary widely, and range from mild fevers to severe life-threatening complications including: hyperparasitemia, hypoglycemia, renal insufficiency, cerebral malaria (CM), severe malarial anemia (SMA), and respiratory distress (Marsh *et al.* 1995; Mockenhaupt *et al.* 2004; Dzeing-Ella *et al.* 2005).

Transmission intensity and the age at which malaria is acquired are important determinants of the clinical manifestations of the disease (Snow *et al.* 1997). However, transmission intensity and age do not adequately explain variation in malaria disease severity among age-matched infants and young children (aged 0 to 3 yrs) with similar levels of parasite exposure and infection rates. Diverse clinical outcomes under these circumstances appear to be conditioned by genetic variability since malaria has exerted significant selective pressure on the human genome, particularly in host-immune response genes that mediate susceptibility and clinical outcomes of the disease (Kwiatkowski 2005).

Macrophage migration inhibitory factor (MIF) is a ubiquitous cytokine produced by T cells (David 1966; Bacher *et al.* 1996), monocytes/macrophages (Calandra *et al.* 1994), and the anterior pituitary gland (Bernhagen *et al.* 1993) in response to pro-inflammatory stimuli. Unlike most cytokines, MIF is constitutively expressed at high levels and stored in preformed vesicles, and therefore, can be rapidly released without *de novo* gene expression (Bernhagen *et al.* 1993;

Bernhagen *et al.* 1998). MIF has potent pro-inflammatory properties and is an important mediator of both innate and adaptive immune responses to bacterial and parasitic infections (Bacher *et al.* 1996; Juttner *et al.* 1998; Bozza *et al.* 1999; Calandra *et al.* 2000; Koebernick *et al.* 2002; Calandra and Roger 2003). Variation in the MIF gene has been shown to influence susceptibility to several inflammatory diseases in non-African populations, including rheumatoid arthritis, atopy, ulcerative colitis, and lung disease (Hizawa *et al.* 2004; Plant *et al.* 2005; Renner *et al.* 2005). To date, five polymorphisms have been identified in the MIF gene, four single nucleotide polymorphisms (SNPs) at positions -173 (G/C), +24 (A/T), +254 (T/C) and +656 (C/G), and a tetranucleotide repeat at -794 ($CATT_{5\text{-}8}$) (Donn *et al.* 2001; Baugh *et al.* 2002; Donn *et al.* 2002). However, only the MIF -173 and MIF -794 polymorphisms have been reported to affect both basal and stimuli-induced MIF production, and influence susceptibility to chronic inflammatory and infectious diseases in Caucasians (Donn *et al.* 2001; Baugh *et al.* 2002; Donn *et al.* 2002; Barton *et al.* 2003; Donn *et al.* 2004; Radstake *et al.* 2005; Renner *et al.* 2005). In addition, high MIF-producing alleles of the -794 CATT repeat were associated with increased susceptibility to high-density parasitemia (HDP, ≥10 000 parasites/μL) in Zambian children with acute malaria (Zhong *et al.* 2005). The role of polymorphic variability in MIF -173 in influencing susceptibility to severe malaria, however, has not been elucidated.

Although elevated MIF levels are associated with enhanced pathogenesis in murine models of malaria (Martiney *et al.* 2000; McDevitt *et al.* 2006), investigations in human malaria have yielded contrasting findings (Awandare *et al.* 2006b; McDevitt *et al.* 2006). Previous investigations showed that MIF production was elevated in intervillous blood during placental malaria (Chaisavaneeyakorn *et al.* 2002; Chaiyaroj *et al.* 2004), thoracic blood vessels of Malawian children with CM (Clark *et al.* 2003), and in peripheral blood from Zambian children

with acute malaria (McDevitt *et al.* 2006). However, we have recently shown that circulating MIF concentrations and peripheral blood leukocyte (PBL) MIF transcripts are suppressed in Gabonese children with mild-to-moderate forms of malarial anemia and hyperparasitemia (Awandare *et al.* 2006b), and in Kenyan children with SMA (Awandare *et al.* 2007b).

To further define the role of MIF in the immunopathogenesis of malaria, we investigated the impact of polymorphic variability at MIF -173 on susceptibility and clinical outcomes of severe malaria, and MIF production. To accomplish these experimental objectives, we performed a cross-sectional, case-control study in a large population of infants and young children with acute malaria (cases) and healthy, aparasitemic individuals (controls). Results presented here describe the relationship between MIF -173 variants and susceptibility to HDP (≥10 000 parasites/μL) and SMA (hemoglobin, Hb<6.0 g/dL). In addition, we describe the functional association between MIF -173 genotypes and circulating MIF levels in children with and without malaria, and MIF production in cultured peripheral blood mononuclear cells (PBMC) stimulated with malarial pigment (hemozoin, *pf*Hz).

### 5.1.4 Study participants and methods

#### 5.1.4.1 *Study site.*

Study participants (n=477) were recruited at the pediatric ward of the Siaya District Hospital (SDH), Nyanza Province, western Kenya. *P. falciparum* transmission in this region is holoendemic with entomological inoculation rates of 100-300 infective bites per annum (Beier *et al.* 1994). Common clinical presentations of severe *P. falciparum* malaria at SDH include HDP and SMA, with CM occurring only in rare cases (Lackritz *et al.* 1992; Ong'echa *et al.* 2006). This area provides a homogenous population for investigating genetic associations with disease susceptibility since >99% of the inhabitants belong to the Luo ethnic group (Ong'echa *et al.* 2006). Additional detail on the study location and manifestations of malaria in the study cohort are presented in our recent publication (Ong'echa *et al.* 2006).

#### 5.1.4.2 *Study participants.*

Study participants (aged 3-36 mos) were enrolled after obtaining written, informed consent from the parents/guardians. The study was approved by the Ethics Committees of the Kenya Medical Research Institute and the University of Pittsburgh Institutional Review Board. Malaria cases (n=363) were recruited from children presenting at SDH for their first hospital contact for the treatment of malaria. Children attending SDH for routine childhood immunizations, free of malaria parasites, afebrile and without history of diarrhea for at least two weeks were enrolled as healthy, aparasitemic controls (AC, n=114). All children were from the Luo ethnic group. HDP was defined using 10 000 parasites/μL as cut-off as per previous studies from the same geographic location (Aidoo *et al.* 2002), and elsewhere (Zhong *et al.* 2005). SMA was defined

as Hb<6.0 g/dL with any parasite density based on previous investigations examining over 10 000 repeated Hb measurements in an age- and geographically-matched cohort from the region of western Kenya where the present studies were conducted (McElroy *et al.* 2000). Only children infected with the *P. falciparum* species were included in this study; those with detectable *P. ovale* or *P. malariae* species were excluded from this study. None of the study participants had CM. Children with prior hospitalizations for any cause were excluded from the study.

#### 5.1.4.3 *Laboratory evaluation.*

Giemsa-stained thin and thick blood smears were used for determination of parasitemia. The number of asexual parasites per 300 leukocytes was obtained and parasites/μL calculated as described previously (Planche *et al.* 2001). Hb concentrations were determined using a Hemocue® system (Hemocue AB, Angelholm, Sweden). HIV-1 status was determined using two serological methods [Unigold™ (Trinity Biotech, USA) and Determine™ (Abbott Laboratories, USA)], and positive serological results were confirmed by proviral DNA PCR as described previously (Otieno *et al.* 2006). All parents/guardians of the study participants received pre- and post-test HIV/AIDS counseling. None of the study participants were receiving antiretroviral drugs at the time of enrollment. Sickle-cell status was determined by alkaline cellulose acetate electrophoresis on Titan III plates (Helena BioSciences, UK) according to the manufacturers' recommendations.

#### 5.1.4.4 *Determination of plasma MIF.*

Before administration of antimalarials and/or any other treatment interventions, venous blood (<3 mL: a volume determined to be safe based on size, weight, and anemia status) was obtained from each study participant as described previously (Ong'echa *et al.* 2006). Concentrations of

MIF in plasma and culture supernatants were determined using an enzyme-linked immunosorbent assay (ELISA) with a matched anti-MIF antibody pair (R&D systems, USA). All samples were assayed at 1:5 and 1:10 dilutions in duplicate, and assays were performed according to manufacturer's recommendations with the limit of detection >62.5 pg/mL.

#### 5.1.4.5 *Genotyping.*

Blood spots were collected on FTA Classic® cards (Whatman Inc., USA) and stored at ambient temperature until DNA isolation. DNA was extracted using the Gentra System (Gentra System Inc., USA). The MIF-173 G/C SNP was genotyped using a Taqman® 5' allelic discrimination Assay-By-Design method (rs755622, Applied Biosystems, USA). The primer sequences were 5'-CGATTTCTAGCCGCCAAGTG-3' (forward) and 5'- AGCAACCGCCGCTAAGC-3' (reverse) while the Taqman "minor groove binder" (MGB) probe sequences were (VIC)5'-AGAACAGGTTGGAGCG-3' and (FAM)5'- AGAACAGCTTGGAGCG-3'. PCR was performed in a total volume of 5 µL with the following amplification protocol: 95°C for 10 minutes, (95°C for 15 seconds, 60°C for 1 minute) × 40 cycles. Following PCR, the genotype of each individual was assigned by measuring allelic specific fluorescence on the ABI Prism® 7900HT sequence detection system using the SDS 2.1® software for allelic discrimination (Applied Biosystems, USA). To validate results obtained with the Taqman® real-time genotyping assays, ~10% of the samples were randomly selected and genotyped using restriction fragment length polymorphism (RFLP) PCR as described previously (Donn *et al.* 2001). There was 100% concordance between the two methods for the samples tested using both methods.

#### 5.1.4.6 *PBMC cultures.*

PBMC were isolated from venous blood obtained from healthy, U.S donors using Ficoll-Hypaque as described previously (Weinberg *et al.* 1981). To ensure complete removal of RBCs, PBMC were treated with RBC lysis buffer (BioWhittaker, USA) for 5 mins and then washed prior to culture. *pf*Hz was isolated from *P. falciparum* (PfD6) parasites cultivated on type O+ RBCs as described in our previous report (Keller *et al.* 2004b). The *pf*Hz preparation was tested for the presence of endotoxin using Limulus amebocyte lysate test (LAL, BioWhittaker, USA), and endotoxin levels were found to be <0.125 U/mL (i.e., <0.025 ng/mL). PBMC were plated at $1 \times 10^6$ cells/mL in Dulbecco's modified Eagles medium (DMEM) containing HEPES buffer (25mM), penicillin (100 U/mL)/streptomycin (100 μg/mL) and 10% heat inactivated human serum from a non-malarious region, and stimulated with media alone (unstimulated control) or a physiological concentration of *pf*Hz (10 μg/mL) as described previously (Keller *et al.* 2004b).

#### 5.1.4.7 *Statistical analyses.*

Kruskal-Wallis tests were used to compare variables across three or more groups, and where significant differences were observed, Mann-Whitney U tests were conducted for pairwise comparisons. To determine associations between MIF -173 genotypes and disease severity, multivariate logistic regression analyses were conducted for each clinical definition (i.e., presence of parasitemia, HDP, and SMA) using a model that controlled for age, sex, HIV-1 status [which included both HIV-1 exposed and HIV-1 PCR(+) results], and sickle-cell trait. Statistical significance for all analyses was determined using a critical alpha value of 0.05.

### 5.1.5 Results

#### 5.1.5.1 *Clinical and parasitological characteristics of study participants.*

Previous studies in Zambian children illustrate that variability at MIF -794 is associated with parasitemic outcomes in children with acute malaria (Zhong *et al.* 2005). To investigate the role of variability at MIF -173 in influencing susceptibility and outcomes of parasitemia, children (n=477; age, 3-36 mos) presenting at a rural hospital with acute malaria or for routine immunizations were stratified according to parasite density: aparasitemic controls (AC, n=114), low-density parasitemia (LDP, <10 000 parasites/μL; n=127), and high-density parasitemia (HDP, ≥10 000 parasites/μL; n=236). The clinical and parasitological characteristics of the study participants upon admission are summarized in Table 6. There were no significant differences in gender distribution among the groups ($P$=0.687). Age was significantly different across the groups ($P$<0.05), largely because children in the AC group were significantly younger than those with HDP ($P$<0.01); the differences in age between the LDP and AC ($P$=0.144) or HDP ($P$=0.236) groups were not significant. Axillary temperature differed across the groups ($P$<0.0001), with children in the HDP group having significantly higher temperatures than those with LDP ($P$<0.005). In addition, Hb concentrations were significantly different across the three groups ($P$<0.001). Children with LDP had lower Hb levels than the HDP group, however, these differences did not reach statistical significance ($P$=0.061). Despite the large disparity in parasite densities between LDP and HDP groups, the proportions of children with SMA (Hb<6.0 g/dL) in these two groups were not significantly different ($P$=0.669). These results illustrate that concomitant peripheral parasite density and SMA are largely independent in children presenting at hospital in this holoendemic area of *P. falciparum* transmission.

**5.1.5.2** ***Distribution of MIF -173 genotypes.***

The genotypic distribution of the MIF -173 G/C polymorphism in aparasitemic controls (n=114) and children with acute malaria (n=363) is shown in Table 7. In the 477 children examined, 19% were GG, 43% were GC, and 38% were CC, representing a significant departure from Hardy Weinberg equilibrium (HWE; $\chi^2$=6.01, $P$<0.01). Proportions of children with malaria from each genotypic group were 80% GG, 78% GC, and 72% CC. The genotypic distribution in aparasitemic controls was 16%, 38%, and 46% for the GG, GC, and CC, respectively. Frequencies of the G and C alleles were 0.35 and 0.65 in aparasitemic controls with no departure from HWE ($\chi^2$=2.70, $P$=0.10). Among children with acute malaria, there were 20% GG, 44% GC, and 36% CC yielding G and C allele frequencies of 0.42 and 0.58, respectively. There was no significant evidence of departure from HWE ($\chi^2$=3.29, $P$=0.075). Chi-squared analysis revealed that there was also no significant difference in the frequency distribution of the MIF -173G/C polymorphism in cases compared to controls ($\chi^2$=2.10, $P$=0.349).

**5.1.5.3** ***Association of MIF -173 genotypic variants with malaria disease outcomes.***

The association between variation at MIF -173 and malaria disease severity was determined by multivariate logistic regression analyses. Parasitemia (*P. falciparum*-positive blood smear), HDP, and SMA were the primary disease outcomes, controlling for age, gender, and sickle-cell status. Since our recent studies also demonstrate that both HIV-1 exposure and HIV-1 virus increase the risk of developing SMA in the current study cohort (Otieno *et al.* 2006), HIV-1 status was also controlled for in the analyses. Relative to homozygous G alleles, the GC and CC genotypes were 10% ($P$=0.861) and 30% ($P$=0.257) less likely to have parasitemia, respectively (Table 8). However, among parasitemic children, the GC and CC genotypes were associated with a 70% ($P$=0.065) and 90% ($P$=0.039) increased risk of developing HDP, respectively,

relative to the GG group (Figure 19 and Table 8). Analyses of the relationship between the MIF -173 polymorphism and SMA (Hb<6.0 g/dL) revealed that children in the GC group had a 30% (*P*=0.307) reduced risk of developing SMA compared to those with the GG genotype, while homozygous C alleles had no impact on the development of SMA in parasitemic children (*P*=0.960; Figure 19 and Table 8). Additional analyses conducted using the WHO definition of SMA (i.e., Hb<5.0 g/dL) (WHO 2000) also failed to yield any significant associations between MIF -173G/C polymorphism and SMA (GC vs. GG, *P*=0.707 and CC vs. GG, *P*=0.967). Taken together, these findings illustrate that the MIF -173 G/C polymorphism is associated with increased susceptibility to HDP, but not SMA, consistent with data presented above (Table 6) demonstrating that parasite density and anemia severity are not significantly associated in this holoendemic *P. falciparum* transmission area.

#### 5.1.5.4 *Functional relationship between MIF -173 G/C polymorphism and circulating MIF levels.*

To examine the functional relationship between the polymorphism and plasma MIF concentrations, aparasitemic controls (n=114) and children with acute malaria (n=363) were analyzed separately since the presence of parasitemia can alter circulating MIF levels (Awandare *et al.* 2006b; Awandare *et al.* 2007b). Among aparasitemic controls, plasma MIF levels were significantly different across the genotypic groups (*P*<0.05, Figure 20). Relative to homozygous G alleles [median (interquartile range), 2179 (1452 – 9341) pg/mL], median circulating MIF concentration was 1.9 times higher in the GC group [4145 (2822 – 6288) pg/mL, *P*<0.05] and 1.7 times elevated in the CC group [3701 (2142 – 5747) pg/mL, *P*=0.322; Figure 20). However, peripheral blood MIF concentrations in children with acute malaria were not significantly

different across the genotypic categories [GG, 4347 (2421 – 7199) pg/mL; GC, 3915 (2045 – 5930) pg/mL; CC, 4085 (2704 – 5936) pg/mL; *P*=0.291, Figure 20].

#### 5.1.5.5 *Influence of MIF -173 G/C polymorphism on MIF production in pfHz-stimulated PBMC.*

Several studies from our laboratory and others have demonstrated that phagocytosis of *pf*Hz is associated with cytokine, chemokine and effector molecule dysregulation *in vivo* (Luty *et al.* 2000; Chaisavaneeyakorn *et al.* 2002; Perkins *et al.* 2003; Awandare *et al.* 2007b), and stimulation of macrophages or PBMC with *pf*Hz *in vitro* elicits a cytokine/chemokine/effector molecule production profile similar to that observed during malaria infection (Pichyangkul *et al.* 1994; Sherry *et al.* 1995; Arese and Schwarzer 1997; Keller *et al.* 2004a; Keller *et al.* 2004b; Ochiel *et al.* 2005; Keller *et al.* 2006a; McDevitt *et al.* 2006). Therefore, to further examine the functional significance of variation at MIF -173, PBMC were cultured from healthy malaria-naïve U.S. individuals with differing genotypes and stimulated with *pf*Hz. As shown in Figure 21, stimulation with physiological concentrations of *pf*Hz (Keller *et al.* 2004b) significantly increased MIF production in individuals with homozygous G alleles ($P<0.05$), while treatment with *pf*Hz significantly decreased MIF production in heterozygous individuals ($P<0.05$, Figure 21). Individuals with homozygous C alleles were not available for these analyses. Taken together, these results demonstrate that variation at MIF -173 is associated with differential MIF production in response to malaria parasite products.

### 5.1.6 Discussion

This study presents the first report on the association between the MIF -173 G/C polymorphism and susceptibility to severe malaria. Distribution of the MIF -173 polymorphism in the Kenyan cohort examined here parallels studies in Zambian children showing a higher frequency of the C allele (Zhong *et al.* 2005). Distribution of the C and G alleles, therefore, differs substantially between sub-Saharan African ethnic groups and Caucasian populations in which the G allele is most prevalent (Donn *et al.* 2001; Donn *et al.* 2002). Differences in allelic frequencies across populations may be due to selective pressure from infectious diseases, such as malaria, that have historically occurred in certain climates and not in others. Consistent with a role of MIF in conditioning outcomes to infectious diseases (Bacher *et al.* 1996; Juttner *et al.* 1998; Bozza *et al.* 1999; Calandra *et al.* 2000; Koebernick *et al.* 2002; Calandra and Roger 2003), multivariate modeling revealed that the CC genotype was significantly associated with an increased risk of HDP, while heterozygosity was associated with a moderately higher risk of developing HDP. However, there was no association between MIF -173 genotypes and SMA, supporting the observation that malarial anemia and parasite burden are not significantly related in this cohort of children. Results presented here showing that parasitemia and the severity of malarial anemia are largely unrelated upon presentation at hospital are consistent with previous studies showing that parasite density during the preceding three months, rather than concomitant parasitemia, predicts the risk of developing childhood SMA in western Kenya (McElroy *et al.* 2000). The G to C transition at MIF -173 creates a potential transcription factor binding site for activator protein (AP)-4, suggesting that polymorphic variability at -173 could functionally alter MIF production (Donn *et al.* 2002). Cloning of a portion of the MIF gene (-775 to +84; excluding the

CATT repeat at -794) into a luciferase reporter vector demonstrated that the -173C promoter was more active in CEM C7A (lung epithelial) cells, while the -173G promoter had the highest activity in A549 (T lymphoblast) cells (Donn *et al.* 2002). These results illustrate the complex relationship between MIF promoter variants and regulation of MIF production. Examination of the functional association between variability at MIF -173 and circulating MIF levels revealed that the C allele was correlated with increased peripheral blood MIF concentrations in aparasitemic controls. These results parallel previous studies in individuals with chronic inflammatory diseases in which the C allele was associated with increased serum MIF concentrations (Donn *et al.* 2002; Donn *et al.* 2004). Circulating MIF levels, however, were not significantly different across the genotypic groups with acute malaria. Of interest, when MIF levels were compared between aparasitemic controls and acute malaria cases (Figure 2), homozygous G alleles were associated with a nearly two-fold increase in MIF levels in parasitemic children, whereas the GC and CC genotypes had similar MIF concentrations in cases and controls. We hypothesize that despite lower baseline MIF production in the GG group, their ability to mount a potent MIF response may aid in controlling parasitemia. This hypothesis is supported by the finding that homozygous G alleles were associated with a decreased risk of developing HDP.

We have previously shown that stimulation of PBMC with *pf*Hz or synthetic hemozoin (sHz) (Awandare *et al.* 2007b) suppresses MIF production, while others have demonstrated that sHz increases PBMC MIF production with specific variants of the MIF -794 polymorphism (5-CATT/5-CATT, 6-CATT/6-CATT and 6-CATT/7-CATT) (McDevitt *et al.* 2006). However, the influence of variation in the MIF gene on MIF production was not determined in our previous studies. Data presented here demonstrate a dichotomous pattern of MIF responses in PBMC

stimulated with *pf*Hz; GG individuals had increased MIF production, while GC individuals had decreased MIF production, suggesting that the pattern of MIF production during malaria is largely influenced by variation at MIF -173. Identical results were obtained using sHz (data not shown), suggesting that the core ferriprotoporphorin IX structure of hemozoin is responsible for altering MIF production, rather than adherent host or parasite-derived proteins, lipids, or nucleic acids. It remains to be determined how individuals with homozygous C alleles at MIF -173 respond to challenge with malarial pigment since these individuals were not available for analyses, largely because of the low frequency of this genotype in Caucasian populations (Donn *et al.* 2001; Donn *et al.* 2002).

Several studies have demonstrated linkage disequilibrium between the MIF -173 SNP and the upstream MIF -794 CATT repeat polymorphism, with haplotypes of the two polymorphic sites being strongly associated with functional gene expression and susceptibility to inflammatory disease (Donn *et al.* 2002; Donn *et al.* 2004; Hizawa *et al.* 2004). Therefore, although not examined in this study, it is possible that some of the relationships between the MIF -173 SNP and malaria disease severity, as well as MIF production may be influenced by the upstream CATT repeat polymorphism. Previous results in reporter constructs, however, demonstrate that variation at -173 alters MIF production in the absence of the -794 CATT polymorphism (Donn *et al.* 2002), suggesting that effects of variation at the two sites may be, at least in part, independent.

Taken together, results presented here illustrate that variation at MIF -173 is associated with functional differences in MIF production and susceptibility to severe malaria. These data further illustrate that MIF -173 variants that confer protection against HDP are also associated with increased MIF production in response to stimulation by malaria parasite products (*pf*Hz).

Given the critical role of MIF in mediating protective immune responses to other infections, including *Salmonella typhii* (Koebernick *et al.* 2002) and *Leishmania major* (Juttner *et al.* 1998), a potent MIF response may be required for effective control of parasitemia during malaria. Since recent studies illustrate that variation in the MIF -794 tetranucleotide repeat is associated with susceptibility to HDP in Zambian children (Zhong *et al.* 2005), we are currently examining the haplotypic distributions of MIF -173 and MIF -794 polymorphisms to obtain additional insight into the role of genetic variation in the MIF gene in conditioning malaria disease outcomes.

### 5.1.7 Acknowledgement

We sincerely thank the parents/guardians and children from the Siaya District community, as well as the U.S blood donors for their participation in the study. We are also grateful to the staff at the Siaya District Hospital, University of Pittsburgh/KEMRI, and University of Pittsburgh laboratories for their contributions to the study. We also thank Dr. Davy Koech, Director of KEMRI, for approving this manuscript for publication.

**Table 6:** ***Demographic, parasitological, and hematological characteristics of study participants.***

| Characteristic | AC | LDP | HDP | *P* |
|---|---|---|---|---|
| Number (*n*) | 114 | 127 | 236 | |
| Gender (*n, %*) | | | | |
| Female | 57 (50) | 60 (47) | 120 (51) | 0.687[a] |
| Male | 57 (50) | 67 (53) | 116 (49) | |
| Age (*mos*) | 10.6 (0.8) | 11.0 (0.5) | 11.5 (0.4)[b] | 0.033[c] |
| Axillary temperature (*°C*) | 37.1 (0.1) | 37.3 (0.1) | 37.8 (0.1) | 0.0001[c] |
| Parasitemia (/*μL*) | 0 | 3,584 (222) | 56,652 (2 869) | <0.0001[d] |
| Geomean parasitemia (/*μL*) | 0 | 1,998 | 39,756 | <0.0001[e] |
| Hemoglobin ( *g/dL*) | 9.9 (0.2) | 6.8 (0.2) | 7.2 (0.1)[b] | <0.001[c] |
| SMA (*n, %*) | NA | 47 (37.4) | 84 (35.5) | 0.669[a] |

Data are presented as mean (SEM) except otherwise indicated.

[a]Chi-Square test; [b]not significantly different from LDP group; [c]Kruskal-Wallis test; [d]Mann-Whitney U test for HDP vs. LDP; [e]Student's t test for HDP vs. LDP.

AC, aparasitemic controls (*P. falciparum*-negative); LDP, low-density parasitemia (<10,000 parasites/μL); HDP, high-density parasitemia (≥10,000 parasites/μL); and SMA, severe malarial anemia (Hb<6.0 g/dL); NA, not applicable.

**Table 7:** ***Genotypic distribution of the MIF -173G/C polymorphism.***

| MIF -173 Genotype | Aparasitemic controls n (%) | Malaria cases n (%) | Total n (%) |
|---|---|---|---|
| GG | 18 (16) | 72 (20) | 90 (19) |
| GC | 44 (38) | 160 (44) | 204 (43) |
| CC | 52 (46) | 131 (36) | 183 (38) |
| | n = 114 | n = 363 | n=477 |
| | P(G) = 0.35 | P(G) = 0.42 | P(G)=0.40 |

P(G) = frequency of G allele.

**Table 8:** ***Association of MIF -173G/C polymorphism with disease susceptibility and severity.***

| MIF -173 Genotype | Parasitemia (*P. falciparum* +) | | | HDP (≥10,000 parasites/μL) | | | SMA (Hb<6.0 g/dL) | | |
|---|---|---|---|---|---|---|---|---|---|
| | **OR** | **95% CI** | ***p*** | **OR** | **95% CI** | ***p*** | **OR** | **95% CI** | ***p*** |
| GG | 1.0 | | | 1.0 | | | 1.0 | | |
| GC | 0.9 | 0.5-1.8 | 0.861 | 1.7 | 1.0-3.1 | 0.065 | 0.7 | 0.4-1.3 | 0.307 |
| CC | 0.7 | 0.4-1.3 | 0.257 | 1.9 | 1.1-3.5 | **0.039** | 1.0 | 0.6-1.9 | 0.960 |

Data presented are results of multivariate logistic regression analyses controlling for age, gender, HIV-1 status, and sickle-cell status. OR, odds ratio; CI, confidence interval.

Association between MIF -173 genotypes and susceptibility to malaria infection (*P. falciparum*-positive blood smear) was examined in 477 children consisting of 114 aparasitemic controls and 363 malaria cases. Analyses of relationships between MIF -173 genotypes and high-density parasitemia (HDP, ≥10 000 parasites/μL), and severe malarial anemia (SMA, Hb<6.0 g/dL) were performed in parasitemic children only (n=363). The GG genotype was used as reference for these analyses since this genotype was considered wild type in previous studies (Donn *et al.* 2001; Donn *et al.* 2002).

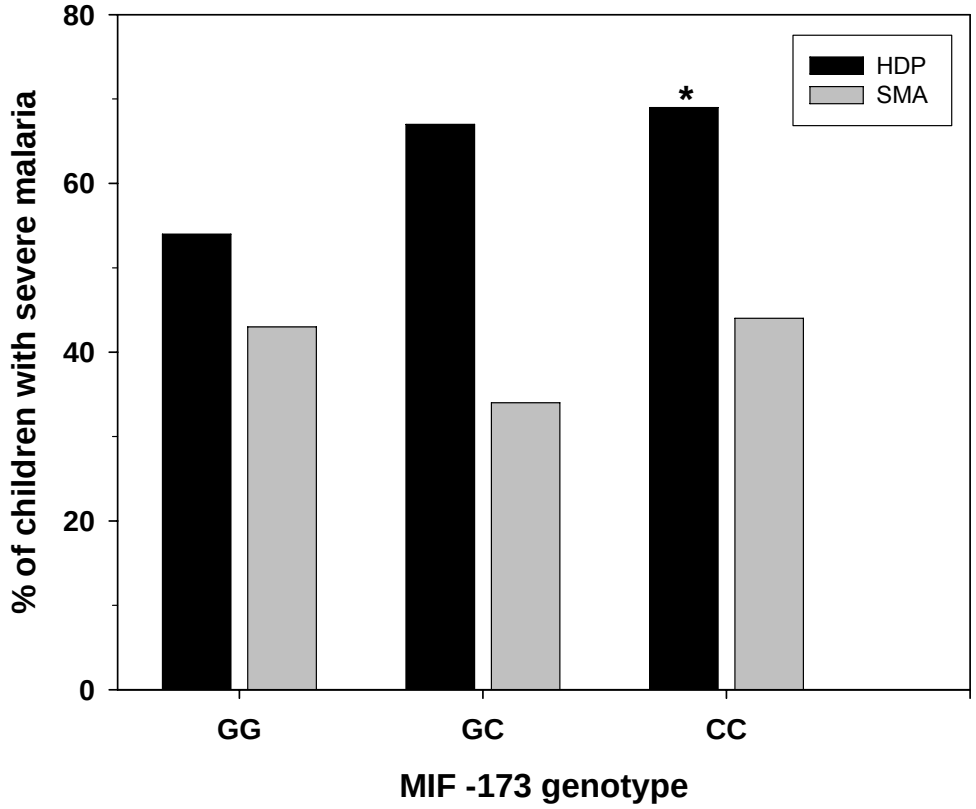


**Figure 19:** ***Proportion of high-density parasitemia and severe malarial anemia stratified according to MIF -173 G/C genotype.***

Proportion of malaria cases with high-density parasitemia, HDP (≥10 000 parasites/μL) and severe malarial anemia, SMA (Hb<6.0 g/dL) are presented for each MIF -173 genotypic category (GG, n=72; GC, n=160; CC, n=130). *Significantly higher compared to the GG group ($P$<0.05), Chi-square test.

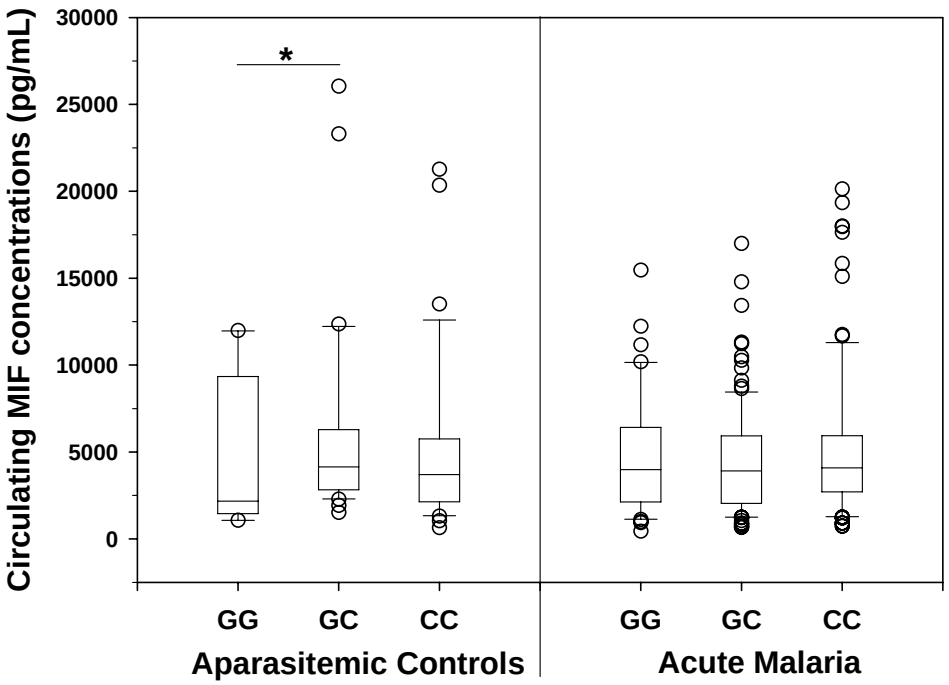


**Figure 20:** ***Circulating MIF levels in the MIF -173 G/C genotypic categories.***

Plasma levels of MIF in aparasitemic controls (GG, n=14; GC, n=32; CC, n=35) and malaria cases (GG, n=50; GC, n=124; CC, n=102) were measured by ELISA and are presented according to MIF -173 genotype. Boxes represent the interquartile range, the line through the box represents the median, whiskers illustrate the $10^{th}$ and $90^{th}$ percentiles, and symbols represent outliers. *Differences between groups were statistically significant by Mann-Whitney U test ($P$<0.05).

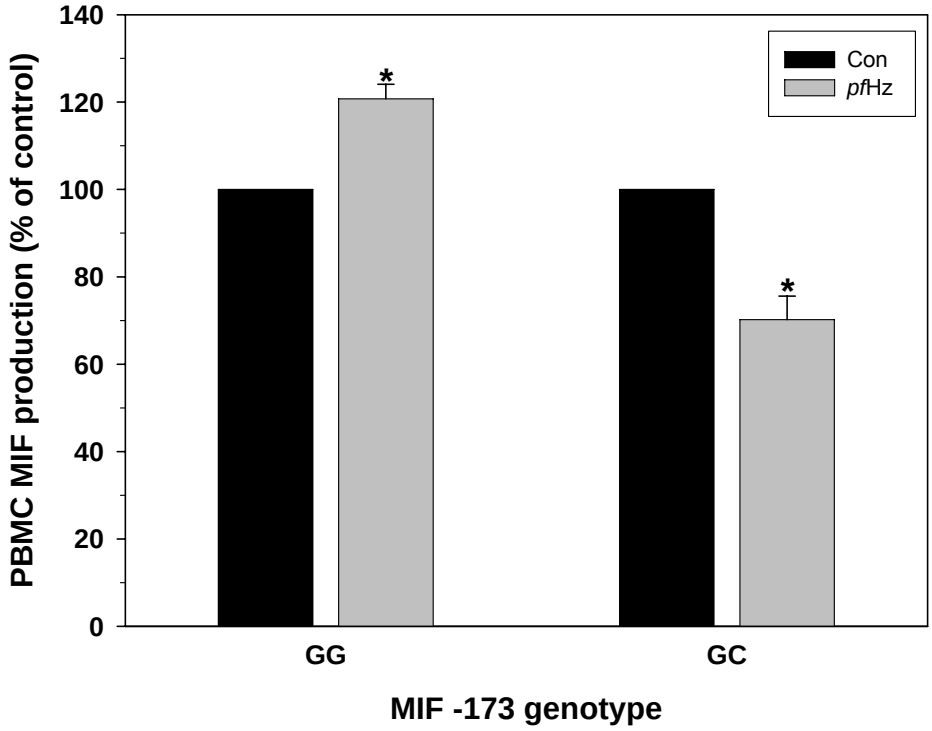


**Figure 21:** ***MIF production in pfHz-stimulated PBMC in the MIF -173 G/C genotypic categories.***

PBMC obtained from healthy, U.S donors with the GG (n=3) and GC (n=3) genotypes at MIF -173 were stimulated with media alone (Con) or a physiological concentration of *pf*Hz (10 μg/mL). MIF concentrations were determined by ELISA in culture supernatants after 48 hours of incubation and are expressed as percent of Con. Data are presented as mean (SEM) for n=3 donors per genotypic group. **P*<0.05 compared to Con, Student's t test.

## 5.2 HYPOTHESES 1 AND 2, PRESENTATION OF MANUSCRIPT ENTITLED: *MACROPHAGE MIGRATION INHIBITORY FACTOR PROMOTER HAPLOTYPES ARE ASSOCIATED WITH SUSCEPTIBILITY TO SEVERE MALARIAL ANEMIA IN CHILDREN.*

*Hypothesis 1: MIF -173 and -794 variants that are associated with decreased MIF production also condition increased susceptibility to SMA.*
*Hypothesis 2: Haplotypes of the MIF -173 and -794 polymorphisms are stronger predictors of SMA susceptibility and MIF production levels than either polymorphism alone.*

To address the second part of *hypothesis 1*, the MIF -794 CATT repeat polymorphism was also examined in the cohort of Kenyan children for whom the MIF -173 SNP had been analyzed. The role of variation at MIF -794 alone in conditioning susceptibility to HDP and SMA and in influencing peripheral blood MIF production was investigated. In addition, *hypothesis 2* was addressed by constructing haplotypes of the MIF -794 and -173 polymorphisms using genotypic data from the loci. The relationships between MIF promoter haplotypes, susceptibility to SMA and HDP, and MIF production were then determined. The results of these investigations are presented in the following unpublished manuscript.

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### 5.2.1 Abstract

Severe malarial anemia (SMA), caused by *Plasmodium falciparum* infections, is one of the leading causes of childhood mortality in sub-Saharan Africa. Recent studies demonstrate that SMA in associated with dysregulated production of the innate immune mediator macrophage migration inhibitory factor (MIF). In addition, we have shown that a functional single nucleotide polymorphism (SNP) at -173 (G/C) of the MIF promoter is associated with increased susceptibility to high density parasitemia (HDP). However, the role of a second functional MIF promoter polymorphism, a $CATT_{5\text{-}8}$ repeat polymorphism at -794, in malarial pathogenesis is largely unknown. To further investigate the influence of MIF genetic variation on susceptibility to SMA, haplotypes of -173 (G/C) and -794 ($CATT_{5\text{-}8}$) polymorphisms were examined in a cohort of Kenyan children (aged 3-31 mos; n=560) residing in a *P. falciparum* holoendemic transmission area. Multivariate regression analyses, controlling for age, gender, HIV-1 status and sickle cell trait revealed that individuals with the MIF -794$CATT_6$/-173G (6G) haplotype were significantly protected from SMA, while carriers of the 7C or 8C haplotypes had a significantly increased risk of developing SMA. Functional analyses demonstrated that carriers of the 7C or 8C haplotypes had significantly lower basal circulating MIF and decreased peripheral blood mononuclear cell (PBMC) MIF production during malaria infection. Taken together, our findings demonstrate that genetic variation in the MIF promoter functionally influences peripheral MIF production and susceptibility to SMA in children.

### 5.2.2 Footnote page

The study was approved by the Ethics Committees of the Kenyan Ministry of Health and the University of Pittsburgh Institutional Review Board, and informed consent was obtained from all participants or the parents/legal guardians of all participating children.

There is no conflict of interest for any of the authors of the manuscript due to either commercial or other affiliations.

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### 5.2.3 Introduction

*Plasmodium falciparum* malaria accounts for 1-2 million deaths annually (WHO 2005). Over 75% of *P. falciparum*-related mortality occurs in sub-Saharan Africa, where malaria accounts for 18% of all deaths in children less than 5 years of age (WHO 2005). Life-threatening complications in children with falciparum malaria include: cerebral malaria (CM), high-density parasitemia (HDP), respiratory distress, and severe malarial anemia (SMA) (Marsh *et al.* 1995; Mockenhaupt *et al.* 2004; Awandare *et al.* 2006a). Clinical presentations of pediatric severe malaria vary markedly across regions with differing transmission intensities, with CM primarily occurring in lower transmission regions and SMA being most prevalent in holoendemic areas (Snow *et al.* 1997). However, differences in disease severity among children of similar ages, residing in a given transmission area are likely due to variation in the host immune response that is conditioned by genetic factors.

Our previous studies, as well as those of others, demonstrate that the nature and magnitude of innate immune mediator production (Perkins *et al.* 2000; Keller *et al.* 2004b; McDevitt *et al.* 2004; Awandare *et al.* 2006a), and genetic variation in host immune response genes (Kwiatkowski 2005) are important determinants of the development and outcomes of severe malaria. Since macrophage migration inhibitory factor (MIF) is a central regulator of innate immune responses to bacterial and parasitic infections (Juttner *et al.* 1998; Bozza *et al.* 1999; Calandra *et al.* 2000; Koebernick *et al.* 2002; Calandra and Roger 2003; Reyes *et al.* 2006), investigations in our laboratory have focused on the role of MIF in malarial pathogenesis. Although studies in murine models of malaria suggest that increased MIF production causes suppression of erythropoiesis and development of SMA (Martiney *et al.* 2000; McDevitt *et al.*

2006), we have recently shown that circulating MIF concentrations progressively decline with increasing anemia severity in Kenyan children with falciparum malaria (Awandare *et al.* 2007b). These results are consistent with our previous findings in Gabon (Awandare *et al.* 2006b) and results by Thuma et. al., (Thuma 2006) showing that peripheral blood MIF levels are significantly lower in children with acute malaria relative to healthy controls.

Of potential importance for influencing patterns of MIF expression and disease severity in children with malaria is genetic variation in the MIF promoter. A tetranucleotide short tandem repeat polymorphism (STRP), MIF-794 $CATT_{5-8}$, and a single nucleotide polymorphism (SNP), MIF-173G/C, functionally influence susceptibility and severity to inflammatory diseases, including arthritis, atopy, lung disease, and scleroderma (Donn *et al.* 2001; Baugh *et al.* 2002; Donn *et al.* 2002; Barton *et al.* 2003; Donn *et al.* 2004; Renner *et al.* 2005). In general, longer CATT repeats (>5) at -794 and the -173 C allele are associated with elevated MIF production and increased susceptibility to inflammatory diseases (Donn *et al.* 2001; Baugh *et al.* 2002; Donn *et al.* 2002; Barton *et al.* 2003; Donn *et al.* 2004; Radstake *et al.* 2005), however, other investigations have observed an opposite pattern of MIF production and disease susceptibility (Donn and Ray 2004; Miterski *et al.* 2004). A previous study in Zambian children revealed that carriers of >5 CATT repeat alleles at -794 had a higher risk of developing HDP relative to those with the 5-repeat allele (Zhong *et al.* 2005). In addition, our recent investigation in a large cohort of Kenyan children demonstrated that a CC genotype at -173 were associated with an increased susceptibility to HDP (Awandare *et al.* 2006c). Although several studies have demonstrated linkage disequilibrium between the polymorphisms at MIF -794 and -173, with haplotypes of the two loci being stronger predictors of disease risk than either one

alone (Donn *et al.* 2002; Barton *et al.* 2003; Donn *et al.* 2004; Hizawa *et al.* 2004), the role of MIF promoter haplotypes in SMA pathogenesis has not been reported.

To comprehensively investigate the relationship between MIF promoter polymorphisms and SMA pathogenesis, we examined the MIF -794 STRP in the cohort of children in whom we recently analyzed the MIF -173 SNP (Awandare *et al.* 2006c), and constructed haplotypes for the two loci. Data presented here show the relationship between malaria disease severity and the -794 STRP, independent of the -173 SNP. In addition, we demonstrate for the first time, an association between MIF -794 and -173 haplotypes, susceptibility to SMA, and peripheral blood MIF production.

### 5.2.4 Study participants and methods

#### 5.2.4.1 *Study site.*

This study was conducted as part of an ongoing study at the Siaya District Hospital (SDH), Nyanza Province, western Kenya, examining the genetic and immunological basis of pediatric SMA (Ong'echa *et al.* 2006). Siaya district is an equatorial community with holoendemic *P. falciparum* transmission area where residents receive up to 300 infective mosquito bites per annum (Bloland *et al.* 1999; Ong'echa *et al.* 2006). The prevalence of falciparum infection is 83% in children 1 to 4 years of age in this region, with SMA and HDP being the primary clinical manifestations of severe childhood malaria (Bloland *et al.* 1999; Ong'echa *et al.* 2006). A more detailed demographic and clinical description of pediatric malarial anemia in this area is presented in our recent publication (Bloland *et al.* 1999; Ong'echa *et al.* 2006).

#### 5.2.4.2 *Study participants.*

Children under three years of age (n=540) presenting at the SDH with acute malaria or for routine immunizations were recruited into the study. All of the study participants were from the Luo ethnic group (Ong'echa *et al.* 2006), thus providing a homogenous population for the genetic studies. After obtaining written informed consent from the parents/guardians, finger-prick blood was collected from study participants for determination of parasitemia and hemoglobin (Hb) levels. Febrile children with detectable *P. falciparum* parasitemia (acute malaria, n=406) were categorized into two groups according to their anemia status: non-SMA (Hb≥6.0 g/dL, n=271), and SMA (Hb<6.0 g/dL, n=135). The definition of SMA was based on the distribution of anemia determined by >10,000 longitudinal Hb measurements in age- and sex-matched children

from the same geographic location (McElroy *et al.* 2000). In addition, the definition of HDP (≥10,000 parasites/μL) was based on the distribution of parasitemia in the population determined in previous studies (Aidoo *et al.* 2002; Ong'echa *et al.* 2006). Healthy children [aparasitemic controls (AC, n=134)] were defined as *P. falciparum*-negative blood smears and free from fever or other symptoms of malaria in the preceding two weeks prior to enrollment. Exclusion criteria included those children co-infected with other species of *Plasmodium*, prior hospitalization or transfusion for any cause, and CM.

#### 5.2.4.3 *Sample collection and laboratory evaluation.*

Prior to administration of anti-malarial therapy and supportive care, venous blood (<3 mL: a volume determined to be safe based on age, size, and anemia status) was collected into tubes containing anti-coagulant. Peripheral blood smears prepared from venous blood were stained with Giemsa reagents and examined for *Plasmodial* parasites under oil immersion. The number of parasites per 300 white blood cells (WBC) was determined, and parasitemia per/μL of blood was calculated using the total WBC count obtained from an automated hematology analyzer (Coulter® A$^c$T diff2™, Beckman Coulter Corp.). Venous blood was also used for a complete blood count, plasma preparation, HIV-1 testing, and where possible, isolation of peripheral blood mononuclear cells (PBMC). Sickle-cell status was determined by alkaline cellulose acetate electrophoresis on Titan III plates (Helena BioSciences, UK) according to the manufacturers' recommendations. HIV-1 status was determined using two serological methods followed by proviral DNA PCR as described previously (Otieno *et al.* 2006). All parents/guardians of the study participants received pre- and post-test HIV/AIDS counseling.

#### 5.2.4.4 *Genetic analyses.*

Blood spots were collected on FTA Classic® cards (Whatman Inc., USA) and DNA was extracted using the Gentra System (Gentra System Inc., USA). Genomic DNA was amplified using the GenomiPhi® system (GE Healthcare, Piscataway, NJ) to obtain sufficient quantities for genetic analysis. Samples were genotyped for the MIF -794 STRP by amplifying a region of the MIF promoter containing the polymorphic site using a pair of flanking primers, one of which was pre-labeled with FAM fluorescent dye. DNA (50 ng) was amplified in a total reaction volume of 25 μL containing 5 pmoles of each primer (forward: 5'-TGCAGGAACCAATACCCATAGG-3', reverse: FAM-5'-AATGGTAAACTCGGGGAC-3') using the Supermix PCR system (Invitrogen, Carlsbad, CA) in a 96-well format on a PTC-100 Peltier thermal cycler (BioRad, Hercules, CA). Forty PCR cycles were carried out, each with denaturation for 30 sec at 95 $^{o}$C, primer annealing for 30 sec at 54 $^{o}$C, and extension for 60 sec at 72 $^{o}$C, followed by a final extension at 72 $^{o}$C for 10 min. The PCR products (estimated size: 340-352 bp) were diluted 1:10 with $H_2O$ and resolved by capillary electrophoresis. Data were analyzed with Genemapper software. Genotyping for the MIF -173 SNP was performed by Taqman® 5' allelic discrimination (Applied Biosystems, USA) and restriction fragment length polymorphism PCR methods according to our previous methods (Awandare *et al.* 2006c).

#### 5.2.4.5 PBMC cultures.

PBMC were isolated from venous blood using Ficoll-hypaque gradient as described previously (Weinberg *et al.* 1981). Cells were cultured at a concentration of 1 x $10^6$ cell/mL (2 x $10^5$ cells/well) in Dulbecco's modified Eagles medium (DMEM) containing HEPES buffer (25mM), penicillin (100 U/mL)/streptomycin (100 μg/mL), and 10% heat inactivated human serum from

malaria-naïve donors (Sigma, St Louis, MO). Cultures were incubated in a humidified atmosphere with 5% $CO_2$ and 5% $O_2$. Supernatants were harvested after 48 hrs of incubation and stored at -80 $^{o}$C until use.

#### 5.2.4.6 *Determination of MIF concentrations.*

MIF concentrations in plasma samples and culture supernatants were determined using an enzyme-linked immunosorbent assay (ELISA) with a matched anti-MIF antibody pair (R&D systems, Minneapolis, MN, USA) according to manufacturer's recommendations. The limit of detection was >31.25 pg/mL.

#### 5.2.4.7 *Statistical analyses.*

Comparison of variables across the three clinical groups (AC, non-SMA and SMA) were conducted using the Kruskal-Wallis test, and where significant differences were obtained, Mann-Whitney U tests were used for pairwise comparisons. Differences in proportions of children within sub-groups, as well as frequencies of genotypes and haplotypes across clinical groups were compared using Chi-square analysis. Circulating and culture supernatant MIF levels between groups were compared using Mann-Whitney U tests. MIF promoter haplotypes were constructed from the -173 G/C and -794 $CATT_n$ genotype data by use of Bayesian Markov Chain Monte Carlo methods, as implemented in the PHASE software program (Stephens and Donnelly 2003). Agreement with Hardy-Weinberg equilibrium was tested using the procedure of Guo and Thompson (Guo and Thompson 1992) as implemented in the software program Arlequin (Excoffier 2005). This program was also used to measure linkage disequilibrium between the -173 and -794 loci, using a likelihood ratio test. For analysis of association between disease and genetic variants, three primary outcomes were defined: presence of parasitemia, HDP, and SMA.

Logistic regression analyses were conducted for each of these outcomes using genotype/haplotype, age, gender, sickle-cell trait, and HIV-1 status as independent predictors.

### 5.2.5 Results

#### 5.2.5.1 *Characteristics of study participants.*

Children (age: 3-32 mos) were categorized into three clinical groups: AC (n=134), non-SMA (n=271), and SMA (n=135). The demographic, clinical, and parasitological characteristics of the children are shown in Table 9. Differences in gender distribution across the groups were not statistically significant ($P$=0.610). However, age differed significantly across the groups ($P$<0.001), with children in the non-SMA group being significantly older than those in the other two groups ($P$<0.001 for both comparisons). Axillary temperature differed across the groups ($P$<0.001) and was significantly elevated in the acute malaria groups relative to the AC group ($P$<0.001 for both comparisons). Hemoglobin, which was used in the categorization criteria, differed across the groups ($P$<0.001) as expected. However, parasitemia, as well as percentage of children with HDP, did not significantly differ between the non-SMA and SMA groups ($P$=0.171, and $P$=0.262, respectively).

#### 5.2.5.2 *Distribution of MIF -794 genotypes and alleles.*

Several studies have demonstrated significant associations of the MIF -794 STRP with various inflammatory diseases in Caucasian and Asian populations (Baugh *et al.* 2002; Barton *et al.* 2003; Hizawa *et al.* 2004). However, the role of this polymorphism in conditioning susceptibility to diseases in African populations remains largely unknown. To investigate the association between variation in the MIF -794 CATT repeat and malarial pathogenesis, frequency distributions of the MIF -794 genotypes and alleles were determined in three groups of children: AC, non-SMA, and SMA. The 5,5 genotype was the most frequent in all three groups

(~ 26-34%), while the 5,8 and 8,8 were the rarest genotypes (<1%, Table 10). The 5,6 genotype differed significantly across the groups (*P*=0.031) and was highest in the non-SMA group. The most prevalent -794 allele in the clinical groups was the 5-repeat (~ 48-53%), while the 8-repeat had the lowest frequencies (0-2%, Table 10). Frequencies of the 6-repeat allele differed significantly across the groups (*P*=0.044), with the non-SMA group having the highest frequency (Table 10), suggesting that this allele may be related to susceptibility to SMA.

#### 5.2.5.3 *Distribution of MIF -794 genotypes and alleles.*

Several studies have demonstrated significant associations of the MIF -794 STRP with various inflammatory diseases in Caucasian and Asian populations (Baugh *et al.* 2002; Barton *et al.* 2003; Hizawa *et al.* 2004). However, the role of this polymorphism in conditioning susceptibility to diseases in African populations remains largely unknown. To investigate the association between variation in the MIF -794 CATT repeat and malarial pathogenesis, frequency distributions of the MIF -794 genotypes and alleles were determined in three groups of children: AC, non-SMA, and SMA. The overall distribution of -794 genotypes in the cohort showed significant departure from Hardy Weinberg equilibrium (HWE, *P*<0.01). A similar departure from HWE was seen by us previously in these samples for the MIF -173 SNP (Awandare *et al.* 2006c), but not for SNPs in the IL-10, TNF-α, and IL-1β promoters (Perkins, unpublished data), suggesting that any departure from HWE in the MIF gene reflects gene-specific events, and not a broader demographic feature of this population that would affect all loci. The 5,5 genotype was the most frequent in all three groups (~ 26-34%), while the 5,8 and 8,8 were the rarest genotypes (<1%, Table 10). The 5,6 genotype differed significantly across the groups (*P*=0.031) and was highest in the non-SMA group (Table 10). The most prevalent -794 allele in the clinical groups was the 5-repeat (~ 48-53%), while the 8-repeat had the lowest

frequencies (0-2%, Table 10). Frequencies of the 6-repeat allele differed significantly across the groups (*P*=0.044), with the non-SMA group having the highest frequency (Table 10), suggesting that this allele may be related to susceptibility to SMA.

#### 5.2.5.4 *Association of MIF -794 genotypes with disease.*

Based on the frequency distribution of the -794 repeat in our cohort (see Table 10), and previous studies demonstrating that an increasing number of CATT repeats are associated with enhanced susceptibility to inflammatory diseases (Baugh *et al.* 2002; Barton *et al.* 2003; Zhong *et al.* 2005), children were divided into three genotypic groups: 5,5, 5,X, and X,X (where X represents alleles with >5 repeats). To investigate the association of the CATT repeat with malaria disease outcomes, multivariate regression analyses were performed with outcome variables as parasitemia, HDP, and SMA, controlling for age, gender, sickle cell trait and HIV-1 status as co-variates. These analyses revealed that relative to individuals with the 5,5 genotype, there was a 57% and 43% increase in the risk of parasitemia in children with 5,X and X,X genotypes (*P*=0.071 and *P*=0.179, respectively, Table 11). The model further illustrated that there was no evidence of an association between genotypic groups and the risk of either HDP or SMA (Table 11). Taken together, these results suggest that an increasing number of CATT repeats at MIF -794 may not be independently associated with malaria disease severity in children residing in a holoendemic transmission area.

#### 5.2.5.5 *MIF promoter haplotypes.*

In addition to the MIF -794 genotypic analyses presented here, we recently demonstrated that variation at MIF -173 was associated with increased susceptibility to HDP in the current cohort of Kenyan children (Awandare *et al.* 2006c). Therefore, to investigate the impact of interactions

between the -794 and -173 loci on susceptibility to severe malaria, haplotypes of the two polymorphic sites were constructed using the PHASE software. In contrast to previous studies from other regions in which the 5C haplotype (i.e., -794 $CATT_5$/-173 C) was rare (Barton *et al.* 2003; Donn and Ray 2004; Hizawa *et al.* 2004), the most common haplotype in the cohort was 5C, while the 8G haplotype had the lowest frequency (Table 12). The distribution of haplotypes showed significant departure from HWE ($P<0.001$). In addition, there was significant evidence of linkage disequilibrium between the -794 and -173 polymorphic sites (likelihood ratio $\chi^2=42.4$, $P<0.001$). Of particular interest, the proportion of 5G to 5C and 6G to 6C differed ~2-fold, while there was a disproportionately higher prevalence of 7C and 8C relative to 7G and 8G (i.e., 9-fold and 7-fold difference respectively, Table 12), suggesting potential interactions between the two polymorphic sites.

#### 5.2.5.6 *Association of MIF promoter haplotypes with disease.*

To determine if MIF haplotypes were associated with susceptibility to malaria, PHASE software was used to analyze the relationship between haplotypes and primary disease outcome variables (i.e., parasitemia, HDP, and SMA). These analyses revealed that MIF haplotypes were significantly associated with SMA ($P=0.02$), but not parasitemia status ($P=0.22$), or HDP ($P=0.83$). Therefore, the association of MIF haplotypes with SMA was further examined by comparing the frequencies of individual haplotypes in the non-SMA vs. SMA groups to identify likely disease-associated haplotypes (Figure 22). The frequency of the 6G haplotype was significantly under-represented ($P=0.031$), while 7C and 8C haplotypes were over-represented in the SMA group relative to children with non-SMA malaria (Figure 22), suggesting possible associations with susceptibility to SMA. Additional analyses using a logistic regression model that controlled for the confounding effects of age, gender, sickle cell trait, and HIV-1 exposure,

demonstrated that children with the 6G haplotype were 51% less susceptible to SMA relative to individuals without the haplotype (*P*=0.001, Table 13). Conversely, children with the 7C haplotype had a 48% increased risk of SMA relative to those without 7C although this difference did not reach statistical significance (*P*=0.132, Table 13). However, when children with 7C or 8C haplotypes were combined, they had a statistically significant 70% increased risk of SMA relative to children without 7C and 8C (*P*=0.035, Table 13). Finally, since children with homozygous C at MIF -173 have a significantly increased susceptibility to HDP (Awandare *et al.* 2006c), it was of interest to know if the risk of SMA was further exacerbated in the subset of children with the -173 CC genotype who carried a 7C or 8C haplotype. Indeed, risk of SMA was nearly 3-fold higher in this subset relative to all other children [odds ratio (95% confidence interval) = 2.90 (1.51-5.57), *P*=0.001], representing a further increase over the risk observed in the 7C/8C haplotype group as a whole. Thus, genetic variation within the MIF promoter can both confer protection from SMA, or condition increased susceptibility to SMA in children infected with *P. falciparum*.

#### 5.2.5.7 *Functional relationship between MIF haplotypes and MIF production.*

To investigate the functional significance of MIF promoter variation in influencing MIF production, the relationship between disease-associated MIF promoter haplotypes and peripheral blood MIF production was examined. Since *P. falciparum* infection dysregulates MIF production (Martiney *et al.* 2000; Awandare *et al.* 2006b; McDevitt *et al.* 2006; Awandare *et al.* 2007b), circulating MIF levels and PBMC MIF production in aparasitemic children and children with acute malaria were examined separately. Circulating levels of MIF were not significantly different between children with and without the 6G haplotype in both the aparasitemic and acute malaria categories (*P*=0.473, and *P*=0.276 respectively, Figure 23A). However, among

aparasitemic children, the 7C/8C haplotype group had significantly decreased MIF levels relative to non-7C/8C children ($P$=0.049, Figure 23B). Possession of the 7C or 8C haplotype did not significantly affect MIF levels in children with acute malaria ($P$=420, Figure 23B).

MIF production in cultured PBMC was also not significantly different in the 6G vs. non-6G haplotype groups in both aparasitemic and acute malaria categories ($P$=0.773, and $P$=0.499 respectively, Figure 24A). On the other hand, while there was no statistically significant difference in PBMC MIF production between children with and without 7C/8C in the aparasitemic category ($P$=0.910, Figure 24B), the 7C/8C haplotype was associated with significantly decreased MIF production in children with acute malaria ($P$=0.018, Figure 24B). Taken together, these data suggest that MIF promoter haplotypes associated with susceptibility to SMA appear to be functionally related to peripheral blood MIF production.

### 5.2.6 Discussion

We recently demonstrated that variation at MIF -173 was associated with susceptibility to HDP, but not SMA, in the cohort of Kenyan children examined here (Awandare *et al.* 2006c). In the current study, a more comprehensive examination of the role of genetic variation in the MIF promoter on conditioning susceptibility to severe childhood malaria was conducted by analyzing the MIF -794 STRP, and by examining haplotypes of MIF -794 and -173. Consistent with previous observations in Zambian children (Zhong *et al.* 2005), the 5-repeat was the most prevalent MIF -794 allele in the Kenyan cohort. This distribution of -794 alleles differs from the patterns reported in Caucasian (Baugh *et al.* 2002) and Asian (Hizawa *et al.* 2004) populations, where the 6-repeat was predominant. Thus, there appears to be significant regional variation in the distribution of the MIF -794 STRP as has been documented for the MIF -173 SNP (Renner *et al.* 2005; Zhong *et al.* 2005; Awandare *et al.* 2006c).

In holoendemic transmission areas, such as our current study site in western Kenya, nearly all of the children experience multiple clinical episodes of malaria during the first five years of life (Snow *et al.* 1997). While a majority of these infected children present with only mild forms of malaria, others experience severe life-threatening complications that predominantly manifest as SMA (Ong'echa *et al.* 2006). Therefore, identifying gene variants associated with susceptibility to severe disease is best accomplished by comparing the genetic backgrounds of *P. falciparum*-infected children who do not develop SMA versus those that present with SMA. The impact of MIF promoter polymorphisms on susceptibility to SMA was investigated by examining MIF gene variants in three clinically distinct groups: healthy, aparasitemic controls; *P. falciparum*-infected children with non-SMA; and children with SMA.

Results presented here demonstrate significant differences in frequencies of the 5,6 genotype, and the 6-repeat allele at MIF -794 across the clinical groups. These differences primarily resulted from an increased representation of the 5,6 genotype and 6-repeat allele in the non-SMA group relative to the SMA group, suggesting that these variants may be associated with a decreased likelihood of developing SMA. However, although a similar pattern was observed for the 6,6 genotype, the differences were not statistically significant, suggesting that the relationship of the 6-repeat allele with susceptibility to SMA should be interpreted with caution.

To account for the confounding effects of other factors that influence the pathogenesis of SMA, additional multivariate regression analysis were conducted to control for age, gender, sickle-cell trait, and HIV-1 status. For these analyses, the 5,5 genotype was used as a reference group since this genotype was the most frequent in the cohort, and because previous studies suggest that differences in disease susceptibility are related to carriage of the 5-repeat (Renner *et al.* 2005). Since data presented here, as well as our previous analyses (Awandare *et al.* 2006c; Ong'echa *et al.* 2006; Ouma *et al.* 2006; Awandare *et al.* 2007b), demonstrate that SMA is not significantly associated with parasite density, HDP and SMA were examined as separate outcome variables. These regression analyses failed to identify a relationship between the presence of >5-repeat alleles and susceptibility to either HDP or SMA. However, there was a trend toward an increased likelihood of being parasitemic (any density) in children with >5-repeat variants, suggesting that children with the 5,5 genotype may be less susceptible to *P. falciparum* infections. Since the parasitemic status of children in areas of high transmission intensity is dynamic, longitudinal studies are required to definitively show an association between MIF genetic variants and susceptibility to infection over extended periods of exposure.

Although the MIF -794 STRP and -173 SNP are independently associated with various diseases (Renner *et al.* 2005), haplotypes of the two polymorphic sites are more strongly associated with functional gene expression and susceptibility to inflammatory disease (Barton *et al.* 2003; Donn *et al.* 2004; Hizawa *et al.* 2004). Therefore, using the genetic data for the two polymorphisms, MIF promoter haplotypes were constructed, and the association of haplotypes with malaria disease severity was investigated. Despite the lack of an independent association between either the MIF -794 STRP or -173 SNP and SMA, haplotypic analyses revealed that the 6G haplotype was protective against SMA, while individuals with either 7C or 8C were at an elevated risk of developing SMA. The haplotypic distribution in the SMA and non-SMA groups showed similar patterns for the 7C and 8C haplotypes, thereby, allowing us to combine the two haplotypes for increased statistical power. Although the 7C haplotype is associated with several inflammatory diseases, including scleroderma (Wu *et al.* 2006), atopy (Hizawa *et al.* 2004) and arthritis (Barton *et al.* 2003), to our knowledge, this is the first report of an association between the 6G haplotype and protection from any disease. Moreover, the role of the 8C haplotype has not previously been examined due to the extremely low frequency of this haplotype in most populations (Donn and Ray 2004; Renner *et al.* 2005). Additional analyses revealed that risk of SMA was further increased in children who had the SMA-susceptible haplotype (i.e., 7C or 8C) in combination with the HDP-susceptible CC genotype at -173 (Awandare *et al.* 2006c), suggesting that the impact of these disease susceptibility traits may be synergistic.

The mechanism(s) by which MIF promoter variants influence susceptibility to SMA was further investigated by examining peripheral blood MIF production in aparasitemic and parasitemic children separately, allowing us to distinguish the impact of genetic variation on basal MIF production from changes that result from host-parasite interactions. While the 6G

haplotype showed no significant relationship with MIF production, carriage of the 7C or 8C haplotypes was associated with decreased MIF production. These observations are in contrast to previous *in vitro* studies in various cell types demonstrating that increased MIF production is associated with an increasing number of CATT repeats (Baugh *et al.* 2002; Donn *et al.* 2004), and in individuals with the 7C haplotype relative to those with non-7C haplotypes (Wu *et al.* 2006). However, results presented here are consistent with other investigations demonstrating that MIF expression is significantly suppressed in 7C promoter constructs compared to 5G and 6G promoters (Hizawa *et al.* 2004), illustrating the complexity of MIF transcriptional regulation. None the less, results presented here showing that the 7C and 8C haplotypes are associated with increased susceptibility to SMA and reduced MIF expression are in agreement with our previous investigations demonstrating decreased MIF production in Kenyan children with SMA (Awandare *et al.* 2007b). These data also support our recent study illustrating that healthy children with a history of severe malaria have lower basal circulating MIF levels relative to those with a history of mild malaria (Awandare 2007a), suggesting that adequate MIF production may be important in the mediating protective immune responses to *P. falciparum* infection.

Taken together, this study represents the most comprehensive investigation to date on the role of MIF promoter polymorphisms in conditioning susceptibility to severe malaria. While the mechanisms by which MIF gene variants mediate protection and pathogenesis of SMA are not fully elucidated, these investigations definitively demonstrate important roles for the 6G, 7C, and 8C haplotypes in the development of SMA. Longitudinal studies are currently ongoing in our laboratories to further examine the mechanisms by which MIF genetic variants influence MIF production and susceptibility to SMA following repeated episodes of malaria.

### 5.2.7 Acknowledgement

Our sincere thanks go to all the parents, guardians, and children from the Siaya District community for their participation in this study. We are also grateful to the staff at University of Pittsburgh/KEMRI laboratories and the Siaya District Hospital for their support during the study. We also thank the KEMRI Director, Dr. Davy Koech for approving this manuscript for publication.

**Table 9:** ***Demographical, clinical, and parasitological characteristics of study participants***

| Characteristic | AC | Acute malaria | | *P* |
|---|---|---|---|---|
| | | Non-SMA | SMA | |
| Number (*n*) | 134 | 271 | 135 | |
| Gender (*n, %*) | | | | |
| Female | 70 (52) | 132 (49) | 61 (45) | 0.610 |
| Male | 64 (48) | 139 (51) | 74 (55) | |
| Age (*mos*) | 8.0 (5.0-14.0) | 11.0 (6.7-16.0) | 8.0 (5.0-12.0) | <0.001 |
| Axillary temperature (*℃*) | 36.9 (36.4-37.7) | 37.6 (36.8-38.7) | 37.5 (36.8-38.3) | <0.001 |
| Parasitemia (/*μL*) | 0 | 27062 (7246-68458) | 37012 (10448-69425) | 0.171 |
| HDP (*n, %*) | - | 190 (70) | 104 (77) | 0.262 |
| Hemoglobin ( *g/dL*) | 10.3 (8.8-11.3) | 8.3 (6.9-9.8) | 5.0 (4.3-5.6) | <0.001 |

Data are presented as median (interquartile range) or n (%), and *P*-values were obtained from Kruskal-Wallis or Chi-square tests respectively, for differences across groups.

**Table 10:** ***MIF -794 genotype and allele frequencies according to clinical categories***

| MIF -794 genotypes | Frequencies, n (%) | | | | *P** |
|---|---|---|---|---|---|
| | **AC** | **Non-SMA** | **SMA** | **Total** | |
| 5,5 | 45 (33.6) | 70 (25.8) | 42 ( 31.1) | 157 (29.1) | 0.226 |
| 5,6 | 38 (28.3) | 102 (37.6) | 35 (25.9) | 175 (32.4) | 0.031 |
| 5,7 | 13 (9.7) | 15 (5.5) | 15 (11.1) | 43 (8.0) | 0.102 |
| 5,8 | 0 (0) | 1 (0.4) | 0 (0) | 1 (0.2) | NT |
| 6,6 | 21 (15.7) | 43 (15.9) | 16 (11.9) | 80 (14.8) | 0.534 |
| 6,7 | 8 (6.0) | 20 (7.4) | 10 (7.4) | 38 (7.0) | 0.856 |
| 6,8 | 0 (0) | 0 (0) | 4 (3.0) | 4 (0.7) | NT |
| 7,7 | 9 (6.7) | 19 (7.0) | 12 (8.9) | 40 (7.4) | 0.746 |
| 8,8 | 0 (0) | 1 (0.4) | 1 (0.7) | 2 (0.4) | NT |
| total | 134 (100) | 271 (100) | 135 (100) | 540 (100) | |
| **MIF -794 alleles** | | | | | |
| 5 | 141 (52.6) | 258 (47.6) | 134 (49.6) | 533 (49.4) | 0.404 |
| 6 | 88 (32.8) | 208 (38.4) | 81 (30.0) | 377 (34.9) | 0.044 |
| 7 | 39 (14.6) | 73 (13.5) | 49 (18.2) | 161 (14.9) | 0.207 |
| 8 | 0 (0) | 3 (0.5) | 6 (2.2) | 9 (0.8) | NT |
| total | 268 (100) | 542 (100) | 270 (100) | 1080 (100) | |

*Chi-square test for frequency distribution across the three clinical groups.

NT, not tested due to small sample (<5) in one or more groups.

**Table 11:** ***Association of MIF -794 genotypic categories with malaria disease.***

| | | Risk relative to 5/5 genotype | |
|---|---|---|---|
| **Disease category** | | **5/X** | **X/X** |
| Parasitemic | | | |
| | Odds ratio | 1.57 | 1.43 |
| | 95% CI | 0.96 – 2.58 | 0.85 – 2.42 |
| | *P* | 0.071 | 0.179 |
| High-density parasitemia | | | |
| | Odds ratio | 1.16 | 1.07 |
| | 95% CI | 0.70 – 1.93 | 0.62 – 1.84 |
| | *P* | 0.562 | 0.818 |
| Severe Malarial Anemia | | | |
| | Odds ratio | 0.74 | 0.92 |
| | 95% CI | 0.43 – 1.25 | 0.52 – 1.63 |
| | *P* | 0.260 | 0.773 |

Children were categorized based on the presence of >5-CATT repeat alleles (X), and odds of disease relative to individuals with the 5,5 genotype was determined by multivariate logistic regression, controlling for age, gender, HIV-1 status, and sickle cell trait.

**Table 12:** ***MIF -794/-173 haplotype frequencies in Kenyan children.***

| MIF -794/ -173 haplotypes | Haplotype frequencies, n (%) |
|---|---|
| 5G | 176 (16.3) |
| 5C | 357 (33.1) |
| 6G | 261 (24.2) |
| 6C | 116 (10.7) |
| 7G | 16 (1.5) |
| 7C | 145 (13.4) |
| 8G | 1 (0.1) |
| 8C | 8 (0.7) |
| total | 1080 (100) |

MIF promoter haplotypes were constructed from -794 and -173 genotypic data using PHASE software.

**Table 13:** ***Association of MIF -794/-173 haplotype carriage and susceptibility to severe malarial anemia (SMA).***

| | Risk of SMA according to MIF -794/-173 haplotype carriage | | |
|---|---|---|---|
| | **6G haplotype** | **7C haplotype** | **7C or 8C haplotypes** |
| Odds ratio | 0.49 | 1.48 | 1.71 |
| 95% CI | 0.31 – 0.77 | 0.88 – 2.48 | 1.04 – 2.82 |
| *P* | 0.002 | 0.132 | 0.035 |

Association with SMA was determined as the odds of SMA in carriers of given haplotypes compared to non-carriers, using multivariate logistic regression controlling for age, gender, HIV-1 status, and sickle cell trait.

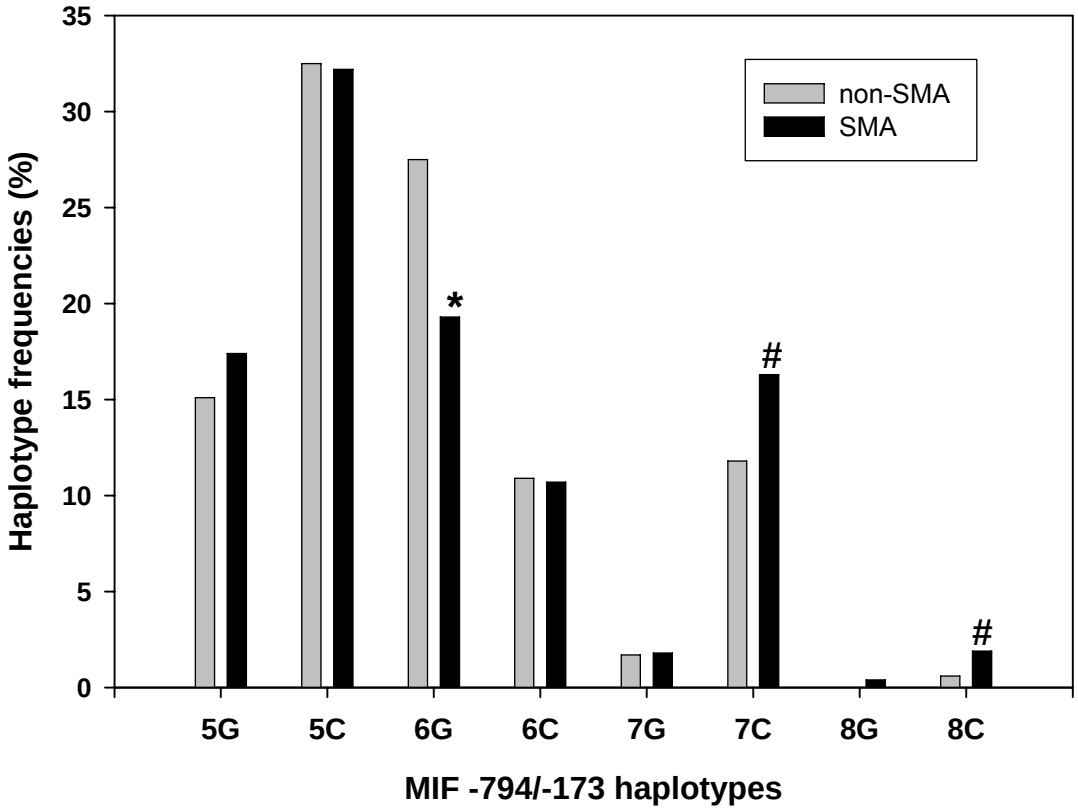
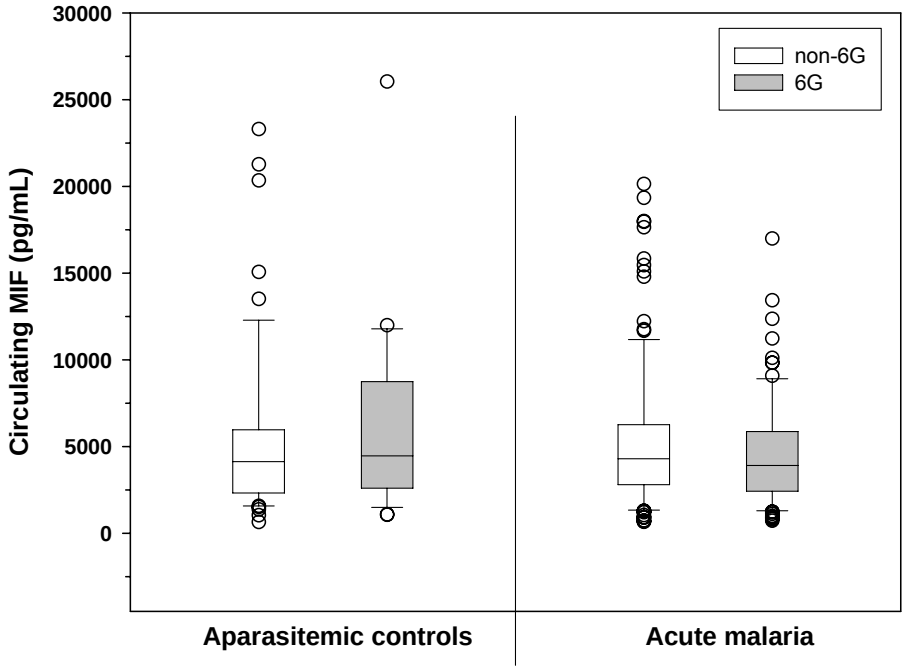



**Figure 22:** ***Association of MIF promoter haplotypes with susceptibility to SMA.***

Haplotypes were constructed from the MIF -794 $CATT_{5\text{-}8}$ and -173 G/C genotypic data using PHASE software, and the frequencies of each haplotype in children with severe malarial anemia (SMA) or malaria without severe anemia SMA (non-SMA) are expressed as percentages. Differences in haplotype frequencies between the two groups were compared using Fisher's test. $^{*}P<0.05$, $^{\#}P<0.10$, compared to the non-SMA group.

**A.**




**B.**

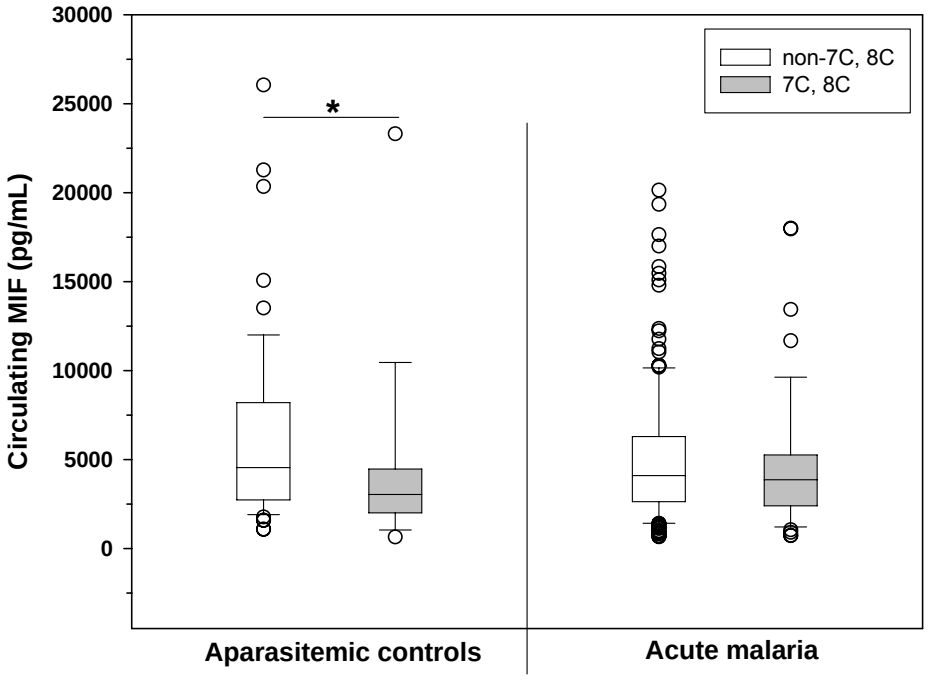
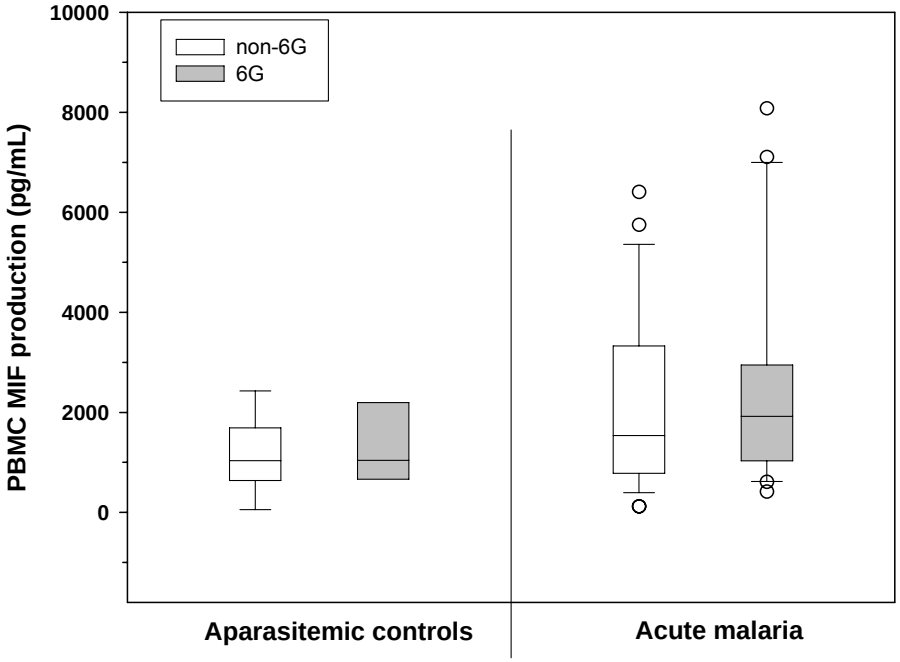



**Figure 23:** ***Functional relationship of SMA-associated haplotypes with circulating MIF levels.***

**Figure 23:** ***Functional relationship of SMA-associated haplotypes with circulating MIF levels.***

Plasma levels of MIF were determined in children with acute malaria (Mal) and aparasitemic controls (AC) using ELISA. **A)** MIF levels in the AC (non-6G, n=53; 6G, n=25) and Mal (non-6G, n=147; 6G, n=95) groups presented according to carriage of the MIF -794/-173 6G haplotype. **B)** MIF levels in the AC (non-7C, 8C, n=59; 7C, 8C, n=19) and Mal (non-7C, 8C n=195; 7C, 8C, n=47) groups presented according to carriage of the MIF -794/-173 7C or 8C haplotypes. Boxes represent the interquartile range, the line through the box represents the median, whiskers illustrate the $10^{th}$ and $90^{th}$ percentiles, and symbols represent outliers. *Differences between groups were statistically significant by Mann-Whitney U test ($P<0.05$).

**A.**




**B.**

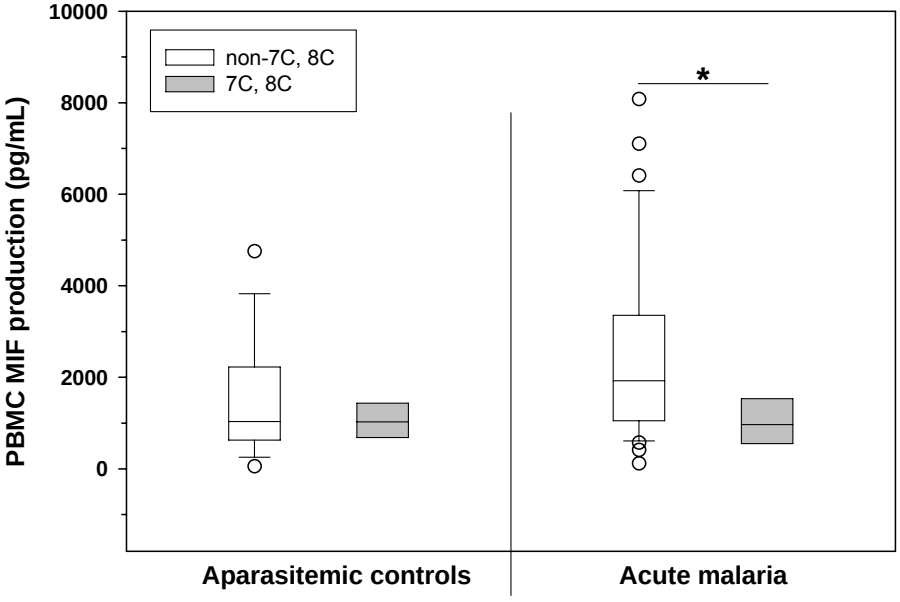



**Figure 24:** ***Functional relationship of SMA-associated haplotypes with peripheral blood mononuclear cell (PBMC) MIF production.***

**Figure 24:** ***Functional relationship of SMA-associated haplotypes with peripheral blood mononuclear cell (PBMC) MIF production.***

Peripheral blood mononuclear cells (PBMC) were isolated from venous blood of children with acute malaria (Mal) and aparasitemic controls (AC) and cultured for 48 hours. Due to limited samples volumes, these experiments were conducted for only children from whom sufficient blood was collected. MIF concentrations in culture supernatants were determined using ELISA. **A)** PBMC MIF production in the AC (non-6G, n=9; 6G, n=8), and Mal (non-6G, n=26; 6G, n=20) categories presented according to carriage of the MIF -794/-173 6G haplotype. **B)** PBMC MIF production in the AC (non-7C, 8C, n=13; 7C, 8C, n=4), and Mal (non-7C, 8C n=38; 7C, 8C, n=8) groups presented according to carriage of the MIF -794/-173 7C or 8C haplotypes. Boxes represent the interquartile range, the line through the box represents the median, whiskers illustrate the $10^{th}$ and $90^{th}$ percentiles, and symbols represent outliers. *Differences between groups were statistically significant by Mann-Whitney U test ($P<0.05$).

# CHAPTER SIX: DISCUSSION

Although *P. falciparum* infections cause a wide range of clinical disease syndromes including cerebral malaria, hypoglycemia, and metabolic acidosis, the majority of malaria-related morbidity and mortality in children is caused by anemia (Breman *et al.* 2001). The mechanisms involved in the pathogenesis of malarial anemia are only partially defined, but appears to be related to the production of inflammatory mediators generated in response to *Plasmodial* infections (Clark and Chaudhri 1988; Perkins *et al.* 2000; Angulo and Fresno 2002; Clark and Cowden 2003; McDevitt *et al.* 2004; Keller *et al.* 2006b). Pro-inflammatory cytokines play a dual role in influencing malaria disease outcomes, such that protective immune responses require induction of IL-12, IFN-γ, TNF-α and NO (Gyan *et al.* 1994; Luty *et al.* 1999; Luty *et al.* 2000; Perkins *et al.* 2000; John *et al.* 2004; Stevenson and Riley 2004; Moormann *et al.* 2006), while over-production of TNF-α, NO, and other potential inflammatory mediators enhance disease pathogenesis (Keller *et al.* 2004b; Awandare *et al.* 2006a; Keller *et al.* 2006a; Grau *et al.* 1989; Kwiatkowski *et al.* 1990; Kurtzhals *et al.* 1998; Akanmori *et al.* 2000; Clark and Chaudhri 1988). For example, while TNF-α and NO have anti-plasmodial activities and are involved in the control of parasitemia (Gyan *et al.* 1994; Kwiatkowski and Perlman 1999), high levels of these inflammatory mediators may also cause anemia by inducing RBC hemolysis and suppressing erythropoiesis (Clark and Chaudhri 1988; Angulo and Fresno 2002; Clark and Cowden 2003; McDevitt *et al.* 2004). Therefore, it appears that a fine balance between protective and pathogenic responses obtained through careful regulation of the sequence and

magnitude at which these inflammatory mediators are produced is required for favorable malaria disease outcomes. Since MIF has been shown to induce both protective and pathogenic immune responses in various diseases primarily by regulating the production of inflammatory mediators including IL-12, IFN-γ, TNF-α and NO (Calandra *et al.* 1994; Calandra and Bucala 1995; Juttner *et al.* 1998; Koebernick *et al.* 2002), we hypothesized that MIF may play a central role in influencing malaria disease outcomes. In support of this hypothesis, studies in murine models of malaria provided strong evidence implicating MIF in the pathogenesis of SMA via suppression of erythropoiesis (Martiney *et al.* 2000; McDevitt *et al.* 2006). Additional evidence for a pathogenic role for MIF in malaria was provided by studies in patients with placental and cerebral malaria demonstrating increased local production of MIF in intervillous blood and chest blood vessel walls respectively (Chaisavaneeyakorn *et al.* 2002; Clark *et al.* 2003; Chaiyaroj *et al.* 2004). However, although children carry the greatest burden of malaria morbidity and mortality (WHO 2005), the role of MIF in the pathogenesis of childhood malarial anemia has remained unknown. In addition, since clinical disease is caused by the blood-stage of the malarial parasite (Kwiatkowski and Perlman 1999), and MIF production during malaria appears to be both tissue- and compartment-specific (Chaisavaneeyakorn *et al.* 2002; Clark *et al.* 2003; Chaisavaneeyakorn *et al.* 2005), systemic regulation of MIF, in addition to local tissue production, may provide important insight into the role of MIF in malarial pathogenesis.

To investigate the role of MIF in regulating cytokine production during childhood malaria, peripheral blood production of MIF, as well as a comprehensive panel of pro- and anti-inflammatory mediators was examined in a cohort of Gabonese children with acute malaria and in healthy aparasitemic controls. These investigations revealed that both plasma MIF and PBMC MIF transcript levels were decreased in children with acute malaria relative to aparasitemic

controls, suggesting that parasitic antigens/products or parasite-induced mediators may be suppressing peripheral blood MIF production. In addition, multivariate regression analyses showed a significant correlation between circulating levels of MIF and IL-12, with IFN-γ emerging as the best predictor of MIF production. These findings are consistent with the fact that IFN-γ induces MIF, and MIF promotes IL-12 production (Calandra *et al.* 1994; Koebernick *et al.* 2002). Interestingly, although MIF can also induce production of TNF-α and NO (Juttner *et al.* 1998), regression analyses presented here found no significant relationship between MIF levels and either TNF-α or NO, suggesting that MIF may not be responsible for the over-production of these mediators in children with acute malaria.

The Gabonese cohort was drawn from an area with hyperendemic malaria transmission; therefore, clinical manifestations of acute malaria included mild malaria, hyperparasitemia and SMA. However, due to the limited sample size of the cohort, a valid analysis of the relationship between MIF production and malaria disease severity could not be conducted. Despite this limitation, the strong correlation between MIF and IL-12 levels in children with acute malaria suggested that peripheral blood MIF production could have implications in regulating disease severity since it is well-established that IL-12 protects against the development of severe malaria (Crutcher *et al.* 1995; Stevenson *et al.* 1995; Luty *et al.* 2000; Perkins *et al.* 2000). As such, we hypothesized that increased MIF production during malaria will protect against the development of childhood SMA.

To comprehensively investigate the role of MIF in the pathogenesis of SMA in children, we examined peripheral blood MIF production in a large cohort of Kenyan children residing in a holoendemic *P. falciparum* transmission area where SMA is the predominant clinical manifestation of severe malaria (McElroy *et al.* 2000; Ong'echa *et al.* 2006). This Kenyan

cohort represents, to our knowledge, the most clinically well-characterized group to-date of children residing in a holoendemic transmission area. This provided the unprecedented advantage of defining clinically distinct categories of children with varying malarial anemia severity, and excluding co-morbidities such as bacteremia and HIV-1, which are common in malaria-endemic areas. In support of a protective role for MIF, these investigations revealed that while children with uncomplicated malaria increased MIF production in response to infection, circulating MIF and PBMC MIF levels progressively declined in children with increasing malarial anemia severity. Furthermore, multivariate regression analyses, controlling for the confounding effects of age and parasite density, showed that circulating MIF concentration was a strong positive predictor of Hb levels, indicating a role for increased MIF in protection against severe anemia. These observations are in contrast to the pathogenic role of MIF that was described in a *P. chabaudi* model of murine malaria in which MIF levels directly correlated with anemia severity (Martiney *et al.* 2000; McDevitt *et al.* 2006). A potentially important difference between MIF regulation in murine versus human systems is that the pituitary gland appears to be the major source of circulating MIF in mice, but not in humans (Calandra *et al.* 1995; Isidori *et al.* 2002). Consistent with this fact, data presented here demonstrate a robust correlation between PBMC MIF gene expression and plasma MIF levels in children, suggesting that PBMC may be the major source of circulating MIF in these children. In addition, the fact that *ex vivo* PBMC MIF production in children with varying anemia severity paralleled the pattern observed with plasma MIF levels demonstrates that *P. falciparum*-derived MIF (Augustijn *et al.* 2007; Cordery *et al.* 2007) did not significantly contribute to circulating MIF in these children. Another significant difference between murine models and childhood malaria is that anemia severity in mice is strongly reflected by parasite density (Martiney *et al.* 2000; McDevitt *et al.*

2006), whereas data presented here showed no significant relationship between SMA and peripheral parasitemia. These differences are likely due to the fact that SMA in mice is associated with rapid increases in parasitemia, accompanied by extensive RBC hemolysis (Martiney *et al.* 2000; McDevitt *et al.* 2006), which is not the case for children examined in the current study who are exposed to holoendemic *P. falciparum* transmission.

In addition to the lack of an association between concomitant parasitemia and SMA, multivariate regression models also revealed that parasite density was not a significant independent predictor of MIF levels, suggesting that parasite burden alone could not be responsible for suppression of MIF in children with SMA. Based on previous studies demonstrating that acquisition of Hz by monocytes and neutrophils plays a role in cytokine dysregulation and disease pathogenesis during acute malaria (Nguyen *et al.* 1995; Luty *et al.* 2000; Lyke *et al.* 2003; Perkins *et al.* 2003; Casals-Pascual *et al.* 2006; Keller *et al.* 2006b), we hypothesized that accumulation of Hz in children with SMA caused suppression of MIF production in circulating phagocytes. Examination of peripheral blood smears revealed a higher abundance of PCM relative to PCN in children with malarial anemia, which is indicative of chronicity of infection since PCN are cleared from circulation within 3-4 days while PCM can persist for more than 10 days (Day *et al.* 1996). More importantly, PCM and PCN levels increased progressively with anemia severity, and PCM emerged as the best predictor of Hb and MIF levels in multivariate regression models than included age and parasite density as co-factors. The robust inverse correlation between MIF and PCM levels was supported by *in vitro* experiments showing that Hz suppressed MIF production from malaria-naïve mononuclear cells, demonstrating that monocyte-acquisition of Hz is, at least in part, responsible for decreased MIF production in children with SMA.

In previous studies implicating MIF in murine SMA pathogenesis, Martiney *et al*. (Martiney *et al.* 2000) proposed that MIF promoted the development of SMA by suppressing erythropoiesis. However, regression analyses presented here found no significant relationship between circulating MIF levels and reticulocyte production in children with acute malaria. Therefore, it was of interest to directly examine the role of MIF in regulating erythropoiesis. Given the difficulties in obtaining bone marrow samples from infants and young children with malarial anemia, a novel *in vitro* model of erythropoiesis was developed using peripheral blood-mobilized CD34+ stem cells to investigate the effects of MIF on erythroid cell development. Analysis of supernatants from erythroid cell cultures demonstrated the novel observation that these cells produced high levels of MIF during erythropoiesis, which is consistent with previous studies showing that erythrocytes store substantial quantities of pre-formed MIF (Mizue *et al.* 2000). Since it is unlikely that erythroid cells will produce and store high levels of a molecule that is detrimental to their survival, we hypothesized that endogenous MIF production may be required for efficient erythropoiesis. However, results presented here show that neutralization of endogenous MIF using blocking antibodies does not have any noticeable adverse effects on erythroid cell proliferation, survival, and maturation, suggesting that endogenous MIF may not be required for efficient erythropoiesis. In addition, although treatment with exogenous MIF slightly augmented proliferation of early erythroid progenitors and marginally suppressed proliferation of more mature erythroid cells, these effects were only minor compared to those observed when cells were cultured in the presence of TNF-α and NO. Furthermore, no significant effects on survival and maturation were observed when erythroid progenitors were cultured in the presence of exogenous MIF, while NO caused increased apoptosis and inhibited maturation of erythroid progenitors. Since data presented here, as well as previous studies, show

over-production of TNF-α and NO in children with acute malaria (Keller *et al.* 2004b; Awandare *et al.* 2006a; Keller *et al.* 2006a; Grau *et al.* 1989; Kwiatkowski *et al.* 1990; Kurtzhals *et al.* 1998; Akanmori *et al.* 2000; Clark and Chaudhri 1988), our findings suggest that TNF-α and NO, rather than MIF, are the major inflammatory mediators involved in suppression of erythropoiesis during malarial infection.

A unique strength of the Gabonese cohort was that extensive longitudinal data were available for the enrollees, including their previous malaria disease history. Using this information, healthy children were selected based on either a history of consistently experiencing mild malaria episodes or a history of exclusively severe malarial episodes. A comparison of MIF production in these two groups revealed an interesting pattern whereby children with prior episodes of severe malaria produced lower levels of MIF. Since these children were aparasitemic and healthy at the time of sampling, we postulated that the observed differences in MIF production levels were likely based on genetic factors. Previous analyses of the MIF promoter sequence identified several putative transcription factor binding sites within the vicinity of two polymorphic loci at -173 (G/C) and -794 ($CATT_{5-8}$) (Paralkar and Wistow 1994; Donn *et al.* 2002; Calandra and Roger 2003), indicating that genetic variation at these loci could functionally alter MIF production. Consistent with such functional variation, the -173 SNP and -794 STRP have been shown to influence MIF production and susceptibility to several inflammatory diseases (Donn *et al.* 2001; Baugh *et al.* 2002; Donn *et al.* 2002; Barton *et al.* 2003; Donn *et al.* 2004; Renner *et al.* 2005). Therefore, we hypothesized that MIF gene polymorphisms conditioned MIF production and SMA susceptibility in children exposed to intense *P. falciparum* transmission. Since factors such as age, sickle cell trait, and HIV-1 status impact on severe malarial pathogenesis, multivariate regression analyses were conducted to

control for the confounding effects of these variables. Independent analysis of the -173 SNP demonstrated that the -173 C allele was dose-dependently associated with susceptibility to HDP, with CC genotypes having a ~2-fold higher risk of HDP compared to the GG genotypes. However, variation at MIF -173 alone was not significantly associated with susceptibility to SMA. Additional investigations demonstrated that PBMC from carriers of the C allele at -173 have decreased MIF production, while GG carriers have increased MIF production in response to Hz stimulation *in vitro*, suggesting that susceptibility to HDP may be related to the magnitude of the MIF response to *P. falciparum* infection. Independent analysis of the MIF -794 STRP revealed no significant relationship between the number of CATT repeats and HDP, SMA or MIF production. Since haplotypes of the MIF -794 and -173 polymorphisms are often stronger predictors of disease susceptibility and MIF production than either locus alone (Barton *et al.* 2003; Donn *et al.* 2004; Hizawa *et al.* 2004), additional investigations examined the relationship of MIF promoter haplotypes with HDP, SMA, and MIF production. Interestingly, despite the lack of independent associations between either the -173 SNP or -794 STRP with SMA, multivariate regression analyses identified carriers of the 6G haplotype to be protected from SMA, while the 7C or 8C haplotypes were associated with a ~2-fold higher risk of developing SMA. In addition, consistent with previous studies showing that 7C promoter constructs produced decreased levels of MIF (Hizawa *et al.* 2004) compared to 5G and 6G haplotypes, data presented here show that carriage of the 7C or 8C haplotypes was associated with lower basal circulating MIF in aparasitemic controls and decreased PBMC MIF production during acute malaria. Taken together, these genetic investigations show a pattern in which decreased MIF production appears to predispose children to severe malaria, suggesting that adequate MIF

production may be an integral part of the protective immune response to *P. falciparum* infection in children exposed to intense transmission.

This study represents the most comprehensive investigation into the role of MIF in childhood malarial pathogenesis. While previous studies in other forms of malaria, such as placental malaria and murine SMA implicated MIF as a promoter of disease pathogenesis (Martiney *et al.* 2000; Chaisavaneeyakorn *et al.* 2002; McDevitt *et al.* 2006), investigations conducted here in a clinically well-characterized cohort of children from a holoendemic transmission area demonstrate that MIF may play a pivotal role in mediating protection against SMA in children. Although the precise mechanism(s) through which MIF mediates protection against SMA remain to be elucidated, data presented here are consistent with a role for MIF in regulating the inflammatory response to *P. falciparum* infection thereby ensuring efficient control of parasitemia while minimizing disease pathogenesis. Based on knowledge gained from all of the investigations conducted in *Specific Aims 1-3*, a proposed model for the role MIF in the pathogenesis of SMA in a holoendemic setting is described in Figure 25. In children with high MIF-producing promoter variants, *P. falciparum* infection induces a robust type 1 immune response characterized by increased production of MIF, IL-12, IFN-γ, and TNF-α. This strong systemic inflammatory response elicits the classical symptoms of acute malaria, including fever, which leads to presentation at hospital with mild forms of malaria. As a result of an early hospital visit, disease resolution is rapid due to the combination of appropriate treatment and a potent anti-parasitic immune response. In the extreme scenario, *P. falciparum* infection elicits only a sub-optimal inflammatory response in low MIF producers which may initially be clinically asymptomatic, or result in non-specific symptoms such as mild fever. In the resource-poor settings in endemic areas, children who do not appear seriously ill are usually administered

anti-pyretic drugs, such as aspirin and acetaminophen, at home and are not immediately taken to the hospital. In such cases, parasitemia persists for a few days leading to accumulation of Hz, which when taken up by monocytes and neutrophils contributes to suppression of MIF production, inhibition of erythropoiesis, and development of anemia. Manisfestation of symptoms associated with severe disease eventually lead to presentation at hospital with a *chronic* infection, characterized by Hb<6.0 g/dL and/or respiratory distress, accompanied by high levels of PCM and decreased MIF production. If given appropriate supportive care, including blood transfusion and administration of oxygen, children with SMA can often achieve full recovery. Prolonged MIF suppression may also contribute to the immuno-suppression associated with malaria (Greenwood *et al.* 1972; Berkley *et al.* 1999) which favors increased parasite replication and susceptibility to co-infections such as bacteremia. The role of MIF in the pathogenesis of SMA proposed here is currently being investigated as part of our longitudinal studies in the Kenyan cohort examining the genetic basis of severe disease susceptibility following repeated episodes of acute malaria.

The results of investigations conducted here have vast public health implications. For example, MIF blocking agents have been proposed as potential treatment for SMA in children based on previous experiments in murine models of malaria demonstrating an association between increased MIF and SMA (Martiney *et al.* 2000; McDevitt *et al.* 2006). However, data presented here represent the first direct investigation of the role of MIF in childhood malaria and demonstrate that the use MIF blocking agents will likely be detrimental in children with SMA. In addition, the search for an effective malaria vaccine has been hampered by the lack of adequate understanding of protective immune responses. Therefore, data presented here significantly increases our understanding of protective inflammatory responses to *P. falciparum*

infection and strongly suggests that induction of a potent MIF response should be an important consideration in the formulation of an effective vaccine against childhood severe malaria.

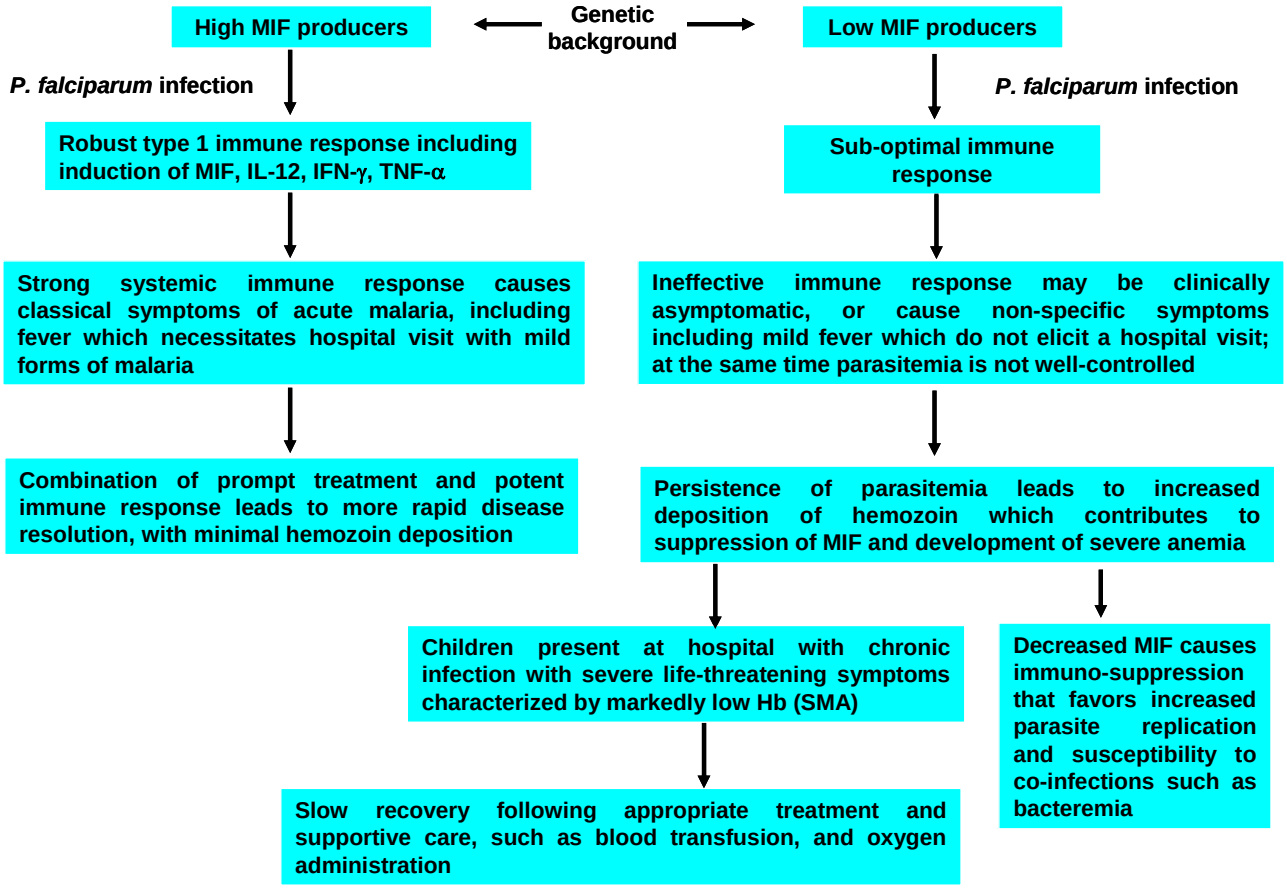



**Figure 25:** ***Proposed model for the role of MIF in the development of SMA in children.***

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